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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE STANDARDISATION OF TUBERCULIN.

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From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

Received for publication August 8th, 1923.

In a previous communication (Eagleton, 1921), a description was given of the method employed at this laboratory for the standardisation of tuberculin.

Briefly stated, this method depends on the specific action of tuberculin on tuberculous animals, the potency of different specimens being compared in this respect with that of a standard tuberculin. A standard was fixed originally by Ehrlich, who showed that tuberculins of the strength adopted were efficient for human and veterinary use.

In 1912 Ehrlich presented to these laboratories a sample of his official Frankfort standard. Upon this our own standard was based, and this has been checked with a sample received from Prof. Kolle of Frankfort in 1921 (*vide* p. 301, Table VII).

The test in fact is essentially that devised by Koch and still used with slight modifications at Frankfort and other places. (Koch, 1891; Otto, 1904; Reeser, 1908; Loewenstein, 1913; Otto and Hetsch, 1921.)

Since our last communication the method has been improved and simplified, large numbers of samples have been tested and re-tested, and several hundred animals employed. We feel that this experience should prove of value to others, and for this reason we propose in this paper to give a *résumé* of the method, a careful consideration of the details and difficulties encountered, and finally a summary of a few of the more important results obtained during the last three years.

DESCRIPTION OF THE TEST.

Sensitisation of animals.—A batch of guinea-pigs is infected with a standardised emulsion of living tubercle bacilli, 0·5 c.c. being injected into the front and inner aspect of the right thigh, deep to the adductor muscles.

The batch is examined week by week, and when the right inguinal glands are easily palpable we proceed to—

The preliminary intracutaneous test.—About the third or fourth week after infection two animals are selected, a small white portion depilated, and into the

skin of each three doses of standard tuberculin injected (0·2 c.c. of dilutions 1/500, 1/1000, 1/2000). This test is repeated weekly until, with the highest dilution of the series, there is obtained next day a strongly positive reaction. We then proceed to—

The main intracutaneous test.—Dilutions of the standard, 1/1000 to 1/8000, are injected as above into the skin of at least two animals, and into corresponding areas of the same animals are injected the same dilutions of the specimen under test. From the severity of the reactions so produced the strength of the sample relative to the standard can be determined. This method is accurate and simple. It is our practice, however, to check all its findings by the—

Subcutaneous method.—This, the original Koch method, is performed as follows:

Preliminary subcutaneous test.—When the animals are sensitive as judged by the intracutaneous test (*vide supra*) this test may be undertaken. One animal of the batch with easily palpable glands is injected with 0·25 c.c. of standard tuberculin subcutaneously. If it survives, or dies at a greater interval than 24 hours, the test is repeated the following week. As soon as 0·25 c.c. kills an animal, three further animals are selected and injected with 0·25 c.c., 0·1 c.c. and 0·1 c.c. of tuberculin respectively. The animal injected with 0·25 c.c. and *one* of those injected with 0·1 c.c. should die overnight. Having obtained this, our "standard reaction," we proceed to—

The main subcutaneous test.—If in the preliminary test the "standard reaction" has been obtained, we proceed on the next day as follows: Each sample that has been found by the main intracutaneous test to be equal to the standard is injected into two animals (0·1 c.c into each). Samples found by the intracutaneous test to be higher or lower than the standard are employed in suitable doses.

The "standard test" consists, then, of a combined intracutaneous and subcutaneous method. The diagram (Table I) shows schematically the testing by this method of a sample which proved equal in strength to the standard.

TECHNICAL DETAILS.

It is not to be thought that the test is essentially a difficult one. We have found, indeed, that with careful attention to detail each factor in the test may be controlled sufficiently to ensure constancy of result.

There are three main factors involved—the animals, the inoculum for sensitisation and the method of dilution and injection.

Animals.

Sufficient animals must be injected to provide for loss from natural causes and to ensure survivors for the cutaneous and subcutaneous test. We find 60 a convenient number for a batch, of which at least one-third should have white or cream areas of skin. They should be young animals and as nearly as possible of the same weight (250 gm.). The use of older animals involves differences in sensitivity owing to certain members of the batch becoming pregnant.

The animals may be "controls" and other survivors from toxin-antitoxin titrations; such "used" animals may be included with fresh stock to form a batch for sensitisation. Previous injections of serum or materials other than tubercle bacilli or their products appear to have no untoward influence on the constancy of the test.

The cutaneous sensitivity to tuberculin of certain animals is apparently increased by a previous injection of horse-serum, so that we often find certain individuals in a batch reacting to a smaller dose of tuberculin than their fellows. This statement, however, only applies to the cutaneous reaction, and the subcutaneous test is not affected. Moreover, animals used for the intracutaneous and von Pirquet test can be kept and used for the subcutaneous injection, provided they are in good condition at the time of the latter. Survivors from the subcutaneous test come, however, into a different category.

Most animals recover from a sublethal dose in five days, and regain their full sensitivity to subsequent injections. This is not, however, invariable, inasmuch as certain animals are rendered refractory for a shorter or longer period, and this variability appears to depend on some idiosyncrasy of the animal. It is noted, moreover, that the more seriously an animal is "shocked" by the first dose, the greater the chance of its remaining insensitive for an abnormally long spell. Thus while animals which have survived one subcutaneous dose may generally be used again after an interval of five days, the results of such tests need great care for their interpretation.

It will be seen in Table II that two animals were unaffected by large doses of sample A. The fact that they subsequently succumbed to the minimal lethal dose of the standard tuberculin proved that it was not due to lack of sensitivity that they failed to respond, but to the weakness of sample A. This reinjection of animals which have survived the first test can thus be employed with advantage as a check on their original sensitivity. Table III, on the other hand, shows the care needed in the interpretation of results. Refractory animals may show merely a large local reaction, with very little, if any, general disturbance. The animals of batch 64 are seen to be of "standard sensitivity," while those of batch 63 are slightly more sensitive. The survivors from these tests were mixed together and used for a second subcutaneous test seven days later. The results are consistent except in the case of three animals, two of whom were seedy but survived when we know they should have died with these doses of sample A. A third animal, not shown on the chart, died from some cause other than the tuberculin injection.

Typical and atypical deaths.—Before we pass on to consider the "atypical" deaths and their chief causes, it is well to emphasise what we mean by a "typical" death (designated in all our work by †).

The animal must die within 24 hours of the tuberculin injection, and show the standard lesions produced in the tuberculous by tuberculin—local œdema, congestion and œdema round the glands affected by the tuberculous process, congestion of any visceral foci that may be present, engorgement of the spleen so marked that in a well-defined case this organ is purplish-black in colour, œdema of the head of the pancreas of such an extent that the duodenal loop of intestine appears to bound a solid mass of jelly. Of these signs the most constant is undoubtedly the condition of the spleen.

Atypical deaths, signified in our tables as X, are of two kinds. The first kind may be described as a late or atypical result of the tuberculin. Thus we frequently find that at the minimal lethal level of a tuberculin one animal may die in a typical manner, while the other dies at the end of 48 hours with less obvious signs of tuberculin reaction (*vide* Table I). Such late deaths are frequent in animals rendered partially refractory by a previous dose of tuberculin. They are also frequent in batches of animals that are dying of Gaertner and other non-specific infections. No animal should be used for the subcutaneous test without a satisfactory examination of its physical fitness. The better in health the tuberculous guinea-pig is, the more constant and typical is its reaction to tuberculin.

Tuberculous animals under good conditions should show a regular increase in weight; they should be active and their muscles firm; their coat should be smooth and glossy and their hair should not fall out. Their appearance indeed should be that of a normal animal, and should a batch of guinea-pigs appear seedy and weak, etc., it is quite certain that some other factor than the tuberculous process itself is at work.

We do not intend to discuss here the chief epidemics with which we have to contend. Suffice it to say that they are chiefly due to two organisms, one associated mainly with enteritis and nervous phenomena—a species of *B. enteriditis* of Gaertner—and a second giving rise to pneumonia, "snuffles" and enteritis, this organism being indistinguishable culturally from *B. fæcalis alkaligenes*. These epidemics are mainly responsible for the second kind of atypical death, which has no connection with tuberculin injection. When once an epidemic due to either or both of these germs is established, very little can be done to control the disaster, but the following palliative measures may be tried. Animals falling sick are immediately removed from the batch; if the death-rate rises to above one or two per day out of a batch of sixty, the batch is carefully examined, all seedy animals removed and the batch transferred to a clean run. Any deficiencies in food supply are remedied as far as possible (*vide infra*). In spite of this, some batches of animals are so hard hit that out of sixty none survive for test purposes.

If, then, an epidemic be established we have little hope of saving many animals of an affected batch.

Our knowledge of the ætiology of these epidemics enables us, however, to take preventive measures. The influence of diet on epidemics among small animals has been noted by many workers, and the value of green food-stuffs was well shown by Glenny and Allen in a paper read before the Pathological Section, Royal Society of Medicine, in November, 1921.

We have ourselves examined culturally animals dying in epidemic and non-epidemic periods, and have found that many apparently healthy animals, even in non-epidemic seasons, carry organisms of the Gaertner or *fæcalis* type in the gut or the ileo-cæcal and mesenteric glands. Of 68 tuberculous animals submitted to careful bacteriological examination after death 30 per cent. died of generalised Gaertner infection, 3 per cent. carried these organisms in the ileo-cæcal and mesenteric glands, *i. e.* were chronically infected, and 10 per cent. in the intestinal contents, and so may be regarded as carriers.

The organisms responsible for the epidemics may then be regarded as

always present, and it is upon the second factor, diet, that we must concentrate.

Tuberculous guinea-pigs should receive a larger ration than non-tuberculous animals, with plenty of green food or swedes; a supply of water is an advantage in hot weather.

They should be kept in large airy runs, on dry bedding (*e.g.* wood-shavings); in cold weather artificial heat must be provided. Given such an environment epidemics are unlikely to occur.

We have dwelt at considerable length on the difficulty of maintaining the health of tuberculous guinea-pigs. For long periods there may be no sign of trouble, but, should the irksome details of thorough routine supervision be relaxed, surely as night follows day disaster will occur.

The Inoculum for Sensitisation.

For this purpose we still use the culture sent us by Dr. Stanley Griffiths. This is a human strain which we have once only passed through a guinea-pig. It is maintained on special medium, which consists of Dorset's egg medium to which is added 5 per cent. glycerine. A three to 5 weeks growth is emulsified by grinding in sterile 0·9 per cent. saline, and standardised to contain 1 mg. of tubercle bacilli, weighed wet, per c.c. Care should be taken that the inoculum is as homogeneous as possible, and it should be shaken thoroughly before being used. We have also used emulsion of tuberculous animal tissue, as practised by Schroeder, at Washington (Schroeder and Brett, 1919), and we generally have one or more tuberculous rabbits available in case by some accident there is no suitable culture for use when required. Such animal emulsions are regular in their infecting power, but generally act slowly, so that the animals take a longer period than usual before they are fully sensitive.

With our standard strain a suitable animal tissue can be obtained by injecting 10 mgm. intravenously into a rabbit and killing the animal 10 days to a fortnight later. The lungs are then homogeneous and solid, and though few tubercle bacilli can be seen, the material is quite satisfactory for infecting the batch.

Method of Dilution and Injection.

With regard to dilution of the tuberculin, there is one point that may be emphasised. Owing to the high glycerine content (5 per cent. for P.T.O. or T.O.A. and 50 per cent. for P.T. or T.) these preparations are very viscous. To avoid any chance of carrying over material from mixtures of higher or lower concentrations, separate pipettes should be used for making each dilution, and great care should be taken to obtain thorough mixture.

Dilutions may be kept in the cold room till next day, but we prefer to use them as soon as they are prepared.

Of the subcutaneous injection we need say little. The injection should be truly subcutaneous and not intraperitoneal. For not only is the minimal lethal dose slightly different in the latter case, but the *post-mortem* appearances are less distinctive.

With regard to the *intracutaneous method* there are several points that require attention.

Preparation of animals.—Each animal selected must undergo a three-fold examination: it must be in good general condition, as was emphasised for the subcutaneous test (animals that are thin, even if showing no other definite signs of disease, are of no use for this test); it must be infected to the same degree as the rest of the batch, as judged by the size of the inguinal glands on the infected side; and lastly, it must have a sufficient area of white or cream colour to allow for the number of injections required.

The areas of skin chosen are first clipped and then depilated by means of a calcium suphide paste, prepared as follows:

To slaked lime is added sufficient water to make a creamy paste. To this add 2 per cent. of its volume of glycerin. Pass H_2S through the paste for 20 minutes to half an hour. Keep in a well-stoppered bottle.

There are one or two details to which attention must be paid. In the first place, only the part necessary should be depilated, the paste should not be left on too long and should be thoroughly removed after the operation, otherwise, owing to the irritant nature of the application, the skin may become inflamed and scratched. The animals should be well dried and kept warm.

Method of injection.—The injection, must be made into and not beneath the skin, the needle inserted bevel upwards, being carried to the same depth each time. We find it convenient to use rustless steel needles and all glass syringes.

Practice is needed if uniformity of injection is to be obtained, and allowance must be made for the variations in thickness of skin in different parts. Thus the skin of the back is very much more tough and thick than that of the abdomen and the depth of the injection must vary accordingly. The quality and size of the resulting lesions are also largely determined by the tension of the surface layers at the place injected, the skin of the abdomen, flanks and back all differing in this respect. For this reason the series of dilutions of tuberculins under test must be injected across the width rather than along the length of the animal, as follows:

A.	B.
Head.	Head.
4 3 2 1	9 5 1
8 7 6 5	10 6 2
12 11 10 9	11 7 3
	12 8 4
Tail.	Tail.

A shows the correct way of placing injections. B shows the incorrect method.

1, 2, 3, 4 = 1/8000, 1/4000, 1/2000, 1/1000 standard.

5, 6, 7, 8,
9, 10, 11, 12, } corresponding dilutions of two samples under test.

The injections should not be made high up on the neck (anterior to the forelimbs), as this part has been found to give very small reactions in certain instances. Injections should not be made near the axillary or inguinal folds, as the material drains away and a smaller reaction results. This is especially well shown in the right inguinal region, where the tuberculous glands can be seen inflamed and sometimes connected with the lesion nearest to hand by a band of red.

There should be a space of about ¾ inch between the injections.

After injection the animal should be returned to a warm place, and if it show excessive signs of cold or shock, a hot bran mash may be given.

Readings are taken at 24 and 48 hours after injection. In most cases the reactions are well seen the next day. In some animals they develop more slowly, and in all cases it is better to read them after 24 and 48 hours.

The lesions vary slightly in size and intensity with the animal employed, and for this reason it is necessary that the dilution of the standard tuberculin be repeated on every animal used. Each animal is "read" independently, the strongest reaction on it being + +, and the remainder graded from this ± ±, +, ±, ∓, −.

Owing to these differences in response we are unable to give an absolute value to any sample, but only its activity relative to the standard.

Two main fallacies may give rise to faulty readings: (1) errors in injection, leaks, etc.: (2) injections into unsuitable areas of skin (*vide supra*) or into old scar-tissue. An animal which has been carefully injected can be read by a novice; an animal which has been carelessly injected will defeat the expert.

The intracutaneous and von Pirquet reactions.—In our previous communication on this subject we showed that by means of the von Pirquet test we could obtain a guide for the subcutaneous test and so save time and animals. During the last three years we have developed the intracutaneous test and gradually abandoned the von Pirquet. All liquid tuberculin made in these laboratories, for human or veterinary use, is tested by the standard method (intracutaneous test checked by the subcutaneous test). Tuberculin that is to be used for von Pirquet or similar diagnostic tests is, in addition, submitted to the von Pirquet test.

For this we still adhere to the "three-scratch test" as described by us formerly, *i. e.* three short parallel scratches on the animal, just deep enough to draw blood in about half a minute.

It is evident that the cutaneous and intracutaneous methods, however modified and improved they may be, depend essentially on the same principles. Every worker obtains the best results with the method which he has himself perfected, and it is hard for him to realise that had he applied his skill to the perfection of another test he might now prefer that to his own.

We still believe, however, that our preference for the intracutaneous method rests not so much on the personal factor as on the relative merits of the two tests:

(1) Whereas in the intracutaneous test we are using dilutions of 1/1000–1/8000, in the von Pirquet test the dilutions range from 1/5–1/40.

(2) The exact amount of material absorbed by any scratch cannot be determined.

(3) The material applied to a scratch can be wiped or licked off.

(4) The "scratch" is not only more irritant at the time of the test, so that the animals may obscure the reactions by extraneous scratching, but the resulting wound heals more slowly than the intracutaneous lesion.

(5) The difficulty of standardising the von Pirquet method is obvious.

Even if any worker succeeds in regulating the length and depth of the

scratches, the variations in thickness of the skin have to be taken into account, and also differences in the method of application of the tuberculin dilutions.

The technique of intracutaneous injection, though more difficult to acquire, is assuredly more suitable for a standard reaction.

THE STANDARD TUBERCULIN.

This is a preparation of undiluted bovine tuberculin. The original standard (S1)* was chosen towards the close of 1919; it was used throughout 1920 and during part of 1921. In the later year its place was taken by a second preparation (S2), which has been in use since. The second standard is as far as possible identical in composition with the first, and is equal to the first in strength as judged by von Pirquet intracutaneous and subcutaneous tests. The latter are shown in Tables IV and V.

The former shows comparative tests on these two preparations at different times. Four different batches of animals were used, one of which was of standard sensitivity (11/11/20), the second (29/9/20) was supersensitive, while the third and fourth were subsensitive. But the two standards are of equal strength as judged by these tests, which confirm the results given by the intracutaneous method.

Table V shows the specific toxicity of S1 and S2 during the time that they have been used as standards.

In this table are included all animals injected with S1 and S2 in the main subcutaneous test. Thus all the animals were sensitive, some were supersensitive, and a few of less than standard sensitivity.

It will be seen that at 0·25 c.c. the death-rate is practically 100 per cent., at 0·1 c.c. practically 50 per cent., and that these figures are reasonably consistent.

The death-rate at 0·05 c.c. and 0·04 c.c. may at first sight appear anomalous, but it must be remembered that only in very sensitive batches are many animals injected with these doses, and that where many animals die at 0·05 c.c. few or none need be injected with 0·1 c.c. The higher doses, 1 c.c. and 0·5 c.c., on the contrary, are only used when the batch is subsensitive, and in such cases we shall find survivals at the 0·25 c.c. level (Table V).

Of the 261 animals injected we see that 69 received 0·25 c.c., and of this number 66 died, *i. e.* 96 per cent.; 114 received 0·1 c.c., and of these 50 died, *i. e.* 43·9 per cent.

The variations from year to year cannot be considered as due to the standard tuberculin but to the sensitivity of the animals, and this for two reasons:

(1) This last year gives as high a death-rate at 0·25 and 0·1 c.c. doses as any preceding year. If variations were due to the tuberculin we should expect a loss of toxicity and not a gain.

(2) Comparisons of the same standards S1 and S2 with other samples (dried and liquid) at different times during the last three years give consistent results.

We may mention that a sample of Koch's tuberculin made many years

* This was equal in strength to Ehrlich's standard (*vide supra*).

before the war was found quite recently by us to be equal in strength to our standard and to tuberculin made by us in 1922 from the strain used originally by Koch.

Owing to the slight differences in sensitivity of batches of animals, even when all precautions are taken, we are unable to give an absolute minimal reacting dose or lethal dose for the standard. We can only say that with a batch of standard sensitivity, the standard tuberculin will give a strong reaction at 1/4000 by the intracutaneous test, at 1/20 and possibly 1/40 by the von Pirquet test, and will kill 100 % of animals at 0·25 c.c. and 50 % at 0·1 c.c.

Samples of unknown strength must be compared with the standard tuberculin, which is the only absolute constant in the test.

With regard to dried preparations, we have tested three since September, 1921. These were all made by precipitation from old tuberculin. They all three appear so far to be stable as judged by periodic comparisons with the liquid standard. In view of the stability of the latter and its convenience, there seems to be little profit in substituting a dry for a wet standard. Moreover the expense would be very considerable compared with the advantage (if any) gained. We are keeping these dried preparations, but at present do not intend to substitute them for S2.

The most interesting thing about them is that although they were all made in the same way, except for details in methods of precipitation, they differed very much in strength, 5 grm. of DT1, 20 grm. of DT2 and 50 grm. of DT3 being required to make 100 c.c. of standard tuberculin respectively (*vide* Table VI). This result seems to have an important bearing on the use of various ophthalmic tuberculins for veterinary and human use. These are wet or dry preparations, many of them made by precipitation of old tuberculin (T or PT) by alcohol. Unless they are standardised, their use is fraught with chance of error.

Our standard for human tuberculin (T) is the same as for bovine tuberculin (PT). Our standard for T.O.A. and for P.T.O. is one-tenth of this.

The ideal standard fulfils the following conditions:

(1) It is stable.

(2) It is reasonably attainable.

(3) It is a true measure of clinical value.

With regard to stability, we have shown that so far as we can judge our standard tuberculin is stable when kept undiluted. Moreover, tuberculins of the same strength can be produced year after year in the laboratory. A comparison of our standard with Frankfort tuberculin of different years is shown in Table VII.

In these laboratories our present standard was adopted for P.T. at the end of 1919. Since then many litres of undiluted tuberculin have been prepared, and all of this has been up to standard strength. The same standard was adopted for human tuberculin in August, 1921, and since then, of the total amount prepared only about 6 % has failed to pass the test.

The bovine culture which we use constantly for the preparation of tuberculin sent by us to another laboratory which had for some time been having trouble with the manufacture of tuberculin. Table VIII shows the test of tuberculin made in that laboratory before and after we sent them our culture

"B 4." We have ourselves experienced a period during which we could not obtain tuberculin from human strains. We found that our cultures of human bacilli, although filming well, yielded only a minute amount of tuberculin as judged by animal test. When we came to test the cultures by injection into rabbits and guinea-pigs we found that they were avirulent. They had become so in process of time, and the exact date of the change could not be determined.* We know, however, that at some period before 1915 they had yielded good tuberculin. Fortunately all strains of tubercle bacilli do not seem to undergo this change. Our best bovine strain has been maintained in artificial culture for at least ten years and still yields tuberculin of standard strength. Koch's original human strain received by us in 1921 yielded tuberculin equal to our standard as soon as we could obtain filming cultures. The possibility of such a change must, however, be borne in mind, and renders imperative constant routine standardisation of all the tuberculin produced.

We may say, then, that given a suitable strain of human or bovine tubercle bacilli, tuberculin can be easily obtained of a strength equal to our standard preparation. We have ourselves used four different strains for tuberculin production during the last two years and all of them were suitable.

In a recent series of tests on tuberculin from various sources two-thirds of the samples were found to be of standard strength. Tests on various samples from England, the Continent and America are shown in Table IX. Most of them were of standard strength. It may be fairly claimed that we have not set our standard too high but that it is reasonably attainable.

The standard satisfies two of the three criteria. It is stable and attainable. Is it a true estimate of clinical value? That is to say, if a tuberculin passes the standard test, is it sufficiently potent for clinical (human and veterinary) use and is it specific? We have no means at this laboratory of testing these points, but we have accumulated records of human and veterinary diagnostic work which are sufficient to convince us that the standard adopted is suitable in these respects. Moreover, as it is founded on the Ehrlich standard and equal in strength to the official Frankfort standard, all the evidence adduced in favour of these preparations may be claimed as its heritage.

Whatever improvement be introduced hereafter, the tests must always depend on the same principle; there will always be the variable factor of specific sensitivity; there will always be the need for a well-tried standard. This we claim to have provided; nor will the inevitable modifications of the future alter the value of the standard which was determined in the past.

In conclusion we have to thank Dr. R. A. O'Brien for the interest he has always taken in the development of this work, which he himself initiated, and our colleague Dr. C. C. Okell for active assistance throughout the last two years.

SUMMARY.

A description is given of the method used in these laboratories for the standardisation of tuberculin.

While it is inevitable that the test will be modified in detail and improved as time goes on, its fundamental principles must remain unchanged.

* The possibility of an error in labelling cannot be absolutely excluded.

This being so, a standard tuberculin is essential, which must be stable, reasonably attainable, and a true laboratory measure of field and clinical potency.

Our standard, based on Ehrlich's original standard, fulfils these conditions.

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TABLE I.—*Standardisation of Tuberculin.*

60 guinea-pigs injected with living T.B. (Emulsion from "glycerin" Dorset or rabbit lung.)

A. After ten days. Preliminary intracutaneous test.
Standard 0·2 c.c. diluted 1/500, 1/1000, 1/2000.

B. Main intracutaneous test (when intracutaneous test 1/2000 = ++*).

	1/1000.	1/2000.	1/4000.	1/8000.
Standard . .	++ .	++ .	± ± .	±
Sample "Q" . .	++ .	++ .	± ± .	±

C. Preliminary subcutaneous test (when intracutaneous test = ++, and with careful selection of guinea-pigs).

Standard 0·25 c.c. = s.
(One week's interval.)
" 0·25 c.c. = †.
" 0·1 c.c. = †.
" 0·1 c.c. = s or x.

D. Main subcutaneous test (when preliminary test complete).

Standard 0·1 c.c. = †.
" 0·1 c.c. = s.
Sample "Q" 0·1 c.c. = †.
" 0·1 c.c. = s.

* The following abbreviations are used in all tables: † = typical death; s = survival; ss = seedy but survived; x = atypical death; ++, ± ±, +, ±, ±, − = degrees of reaction; n = no test.

TABLE II.—*Value of Using Survivors from Subcutaneous Test.*

Date.	Dose.	Standard.	Sample A.
30.6.20	0·5 c.c.	†	s
	0·25 c.c.	†	s
	0·1 c.c.	†	s
1.7.20	0·1 c.c.	†	n
	0·05 c.c.	ss	n
	0·5 c.c.	n	s
8.7.20	2·0 c.c. (guinea-pig A)	n	s
12.7.20	1·5 c.c. (,, B)	n	s
	0·1 c.c. (,, A)	†	
(same animals as on 8.7.20)	0·1 c.c. (,, B)	†	

TABLE III.

	Dose.	Standard.	Sample A.	Sample B.	Sample C.	Sample D.	Sample E.	Sample F
Batch 63—								
24.5.23	0·25 c.c.	†	—	—	—	—	—	—
	0·1 c.c.	† †	† † †	† s	†	—	†	—
	0·05 c.c.	s s	s s s	s	†	—	s	—
Batch 64—								
25.5.23	0·25 c.c.	†	—	—	—	—	—	—
	0·1 c.c.	† ss	s s	s s x	—	—	—	† s
	0·05 c.c.	s	—	—	† s	—	—	—
Survivors from Batches 63 and 64—								
1.6.23	0·25 c.c.	†	ss	†	†	†	—	—
	0·1 c.c.	† s	ss s	† s	s s	† s	—	—
	0·05 c.c.	s	s	s	—	s	—	—

Samples A, B, D, E and F were, on the intracutaneous test, equal to the standard.

TABLE IV.—*Subcutaneous Tests in Parallel on Standards S1 and S2.*

Dose.	11.11.20.		29.11.20.		9.2.21.		19.2.23.	
	S1.	S2.	S1.	S2.	S1.	S2.	S1.	S2.
0·5 c.c.	—	—	—	—	—	—	†	†
0·25 c.c.	†	†	—	—	†	†	† †	† †
0·1 c.c.	ss	†	—	—	s	s	s s	s s
0·05 c.c.	s	s	—	—	—	—	s	s
0·04 c.c.	n	n	† †	† x	—	—	—	—
	—	—	s s	s s	—	—	—	—

TABLE V.—*Subcutaneous Tests on Two Standards* 1919–1923 (261 *animals used*).

Dose.	S1, 1919. †	Total injec.	% †	S1, 1920. †	Total injec.	% †	S2, 1921. †	Total injec.	% †	S2, 1922. †	Total injec.	% †	S2, 1923. †	Total injec.	% †
1·0 c.c.	1	1	100	—	—	—	—	—	—	—	—	—	—	—	—
0·5 c.c.	12	12	100	—	—	—	—	—	—	1	1	100	1	1	100
0·25 c.c.	17	18	94	4	4	100	24	25	92	15	15	100	6	6	100
0·15 c.c.	—	—	—	—	—	—	3	4	75	—	—	—	—	—	—
0·1 c.c.	11	20	55	6	11	54	7	18	40	13	30	43	9	17	53
0·05 c.c.	5	8	62·5	7	15	47	7	18	40	4	11	36	0	5	0
0·04 c.c.	—	—	—	2	4	50	2	4	50	—	—	—	—	—	—
0·025 c.c.	0	6	0	0	1	0	0	2	0	0	3	0	—	—	—

TABLE VI.—*Comparison of Three Dried Tuberculins—DT1, DT2, DT3 with S2.*

By intracutaneous tests DT1 (5%) = S2 Sept., 1921, and Feb., 1922.
„ „ „ DT2 (20%) = S2 May, 1922, and Jan., 1923.
„ „ „ DT3 (50%) = S2 Jan., 1923.

Subcutaneous Test.

Dose.	Sept., 1923. DT1 5%.	S2.	May, 1922. DT2 20%.	S2.	Feb., 1923. DT3 50%.	S2.
0·5 c.c.	—	—	†	—	†	†
0·25 c.c.	—	—	†	†	† s	† †
0·1 c.c.	†	†	s	†	s ss	ss s
0·05 c.c.	† † s s	† †	—	—	—	—
0·04 c.c.	† s	s	—	—	—	—
0·025 c.c.	s s	s	—	—	—	—

TABLE VII.—*Comparison of S1 and S2 with Ehrlich Original Tuberculin (= Eh.) and Frankfort Standard of* 1921 (= *Fr.*).

Date.	Dose.	Subcutaneous test. S2.	Eh.	Fr.	Result of intracutaneous test.
23 . 9 . 21	—	—	—	—	S1 = Eh.
17 . 10 . 21	0·25 c.c.	†	†	—	—
	0·1 c.c.	†	ss	—	—
	0·05 c.c.	ss	†	—	—
1 . 11 . 21	—	—	—	—	S2 = Eh.
18 . 1 . 22	0·25 c.c.	†	—	—	—
	0·1 c.c.	† s	† s	—	—
	0·05 c.c.	—	s s	—	—
24 . 1 . 22	—	—	—	—	Fr. = S2 (also von Pirquet Fr. = S2)
1 . 2 . 22	0·2 c.c.	†	—	†	—
	0·1 c.c.	—	—	† †	—
	0·05 c.c.	—	—	s	—

TABLE VII—*continued.*

Date.	Dose.	Subcutaneous test. S2.	Eh.	Fr.	Result of intracutaneous test.
6 . 2 . 23	—	—	—	—	Fr. = S2
14 . 3 . 23	—	—	—	—	Fr. = S2
24 . 5 . 23	0·25 c.c.	†	—	—	—
	0·1 c.c.	† †	—	† s	—
	0·05 c.c.	s s	—	s	—
25 . 5 . 23	0·25 c.c.	†	—	—	—
	0·1 c.c.	† ss	—	s s x	—
	0·05 c.c.	s	—	—	—
1 . 6 . 23	0·25 c.c.	†	—	†	—
	0·1 c.c.	† s	—	† s	—
	0·05 c.c.	s	—	s	—

TABLE VIII.—*Tuberculin from Laboratory A.*

Date.	Dose.	S1.	S2.	Laboratory A.
27 . 10 . 19	0·5 c.c.	†	—	s
	0·25 c.c.	†	—	s
	0·1 c.c.	s	—	s
30 . 6 . 20	0·5 c.c.	†	—	s
	0·25 c.c.	†	—	s
	0·1 c.c.	†	—	s
1 . 7 . 20	0·5 c.c.	—	—	s
	0·1 c.c.	†	—	—
	0·05 c.c.	s	—	—
8 . 7 . 20	1·5 c.c.	—	—	s
	2·0 c.c.	—	—	s
25 . 5 . 23	0·25 c.c.	—	†	By intracutaneous test = S2 (after receiving B4)
	0·1 c.c.	—	† ss	s s
	0·05 c.c.	—	ss	—
1 . 6 . 23	0·25 c.c.	—	†	†
	0·1 c.c.	—	† s	† s
	0·05 c.c.	—	s	—

Note apparent discrepancy between intracutaneous and subcutaneous tests 25 . 5 . 23.

TABLE IXA.—*Comparison against Standards of Two Commercial "Bovine" Tuberculins, N1 and N2, issued from a Laboratory in Different Years.*

Date.	Dose.	Subcutaneous test. S1.	S2.	N1.	N2.	Other tests.
23 . 11 . 20	0·5 c.c.	—	—	†	—	By von Pirquet N1 = ½ S1
	0·25 c.c.	†	—	† †	—	—
	0·1 c.c.	†	—	s	—	By intracutaneous N1 = ½ S2
	0·05 c.c.	† †	—	s	—	—
	0·025 c.c.	ss	—	—	—	—
24 . 5 . 23	—	—	—	—	—	By intracutaneous N2 = S2
25 . 5 . 23	0·25 c.c.	—	†	—	—	—
	0·1 c.c.	—	† ss	—	† †	—
	0·05 c.c.	—	s	—	—	—

Note.—N1 is only half strength; N2 = standard.

TABLE IXB.—*Comparison against Standards of Tuberculins P1, diluted for Veterinary Use, and P2, undiluted.*

Date.	Dose.	S1.	S2.	P1.	P2.
6 . 12 . 20	2·0 c.c.	—	—	—	—
	1·0 c.c.	—	—	†	—
	0·75 c.c.	—	—	† †	—
	0·5 c.c.	—	—	s s	—
	0·1 c.c.	†	—	—	—
	0·05 c.c.	† † † x s	—	—	—
	0·025 c.c.	s	—	—	—
25 . 5 . 23	0·25 c.c.	—	—	—	—
	0·1 c.c.	—	† ss	—	† s

Note.—P1 is only 1/15 instead of 1/10 strength of standard, while P2 = standard.

TABLE IXC.—*Tests on Veterinary Tuberculin from Laboratory D.*

Date.	Dose.	S2.	D1.
6 . 2 . 23	—	—	By intracutaneous test $= \frac{S}{20}$
25 . 5 . 23	0·25 c.c.	†	—
	0·1 c.c.	† ss	—
	0·05 c.c.	s	—
	2·0 c.c.	—	†
	1·0 c.c.	—	s

Note.—D1 is only 1/20 standard instead of 1/10.

TABLE IXD.—*Tests on Tuberculins from Laboratory R, R1 (diluted for Veterinary Use) and R2 (undiluted).*

Date.	Dose.	S2.	R1.	R2.
24 . 5 . 23	—	—	By intracut. test $= \frac{S}{10}$	—
18 . 4 . 23	—	—	—	By intracut. test = S
3 . 5 . 23	0·25 c.c.	†	—	—
	0·1 c.c.	† x † s	—	† s

TABLE IXE.—*Tests on Tuberculins from Laboratory A.*

Date.	Dose.	S2.	A1.	A2.	A3.	A4.
29 . 5 . 22	—	—	By intracut. test = S2	—	—	—
4 . 7 . 22	0·2 c.c.	†	†	—	—	—
	0·1 c.c.	ss	†	—	—	—
	0·1 c.c.	† †	s*	—	—	—
	0·05 c.c.	† s	† †	—	—	—
	0·025 c.c.	—	s	—	—	—
3 . 23	0·25 c.c.	†	—	By intracut. test = S2	—	—
	0·1 c.c.	† †	—	†	s s	† s
	0·05 c.c.	s s	—	†	—	—

Note.—A1 and A4 = standard, A2 is stronger, A3 weaker.

* *Note.*—Survival out of order; animal not sensitive.

TABLE IXF.—*Tests on Various Tuberculins from Laboratory B.*

Date.	Dose.	S2.	B1.	B2.	B3.	B4.	B5.
10.11.21	—	—	By von Pirquet test = S2	—	—	—	—
24.1.22	—	—	By von Pirquet test = S2	—	—	—	—
29.5.22	—	—	By von Pirquet test = S2	—	—	—	—
31.1.22	0·2 c.c.	†	†	—	—	—	—
	0·1 c.c.	ss	†	—	—	—	—
	0·1 c.c.	† †	†	—	—	—	—
	0·05 c.c.	† ss	† ss	—	—	—	—
	0·015 c.c.	—	s	—	—	—	—
3.23	0·25 c.c.	†	—	By intracut. test = >S2	—	—	—
24.5.23	0·1 c.c.	† †	—	† †	—	—	—
	0·05 c.c.	s s	—	s s	—	—	—
25.5.23	0·25 c.c.	†	—	—	—	—	—
	0·1 c.c.	† ss	—	—	s s	s s	†
	0·05 c.c.	s	—	—	—	—	s

TABLE IXG.—*Tests on Tuberculins from Laboratory C, prepared in successive years.*

Date.	Dose.	S2.	C1922.	C1923.
28.6.22	—	—	By intracut. test = S2	—
4.7.22	0·1 c.c.	†	†	—
	0·05 c.c.	ss	s	—
10.4.23	—	—	By intracut. test = S2	—
25.5.23	0·25 c.c.	†	—	—
	0·1 c.c.	† ss	—	† s
	0·05 c.c.	s	—	—

THE PLATELET-COUNT IN RATS SUFFERING FROM VITAMIN-A DEFICIENCY.

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In a recent communication on this subject by Cramer, Drew and Mottram (1923), an attempt has been made to explain the divergence between their findings and ours with respect to the platelet content of the blood of rats suffering from a vitamin A deficiency. Briefly stated the position is as follows: Cramer, Drew and Mottram (1922) produced evidence to show that the deprivation of young growing rats of the fat-soluble vitamin resulted in a severe thrombopœnia. This diminution in the platelet-count—from 1,000,000 to 300,000 or even less per c.c.—was found by them to be such a constant and characteristic feature of vitamin A deficiency in the rat that they termed it the specific lesion of this condition, and based on this observation an explanation of the diminished resistance to infection shown by rats suffering from such a deficiency. With a view to employing this reduction in platelets as a measure of the degree of vitamin A deficiency we repeated this work, and obtained results which were not in keeping with the findings of Cramer, Drew and Mottram (Bedson and Zilva, 1923). In our experience the withholding of vitamin A from the diet of growing rats does not result in a severe thrombopœnia. Counts made on rats in the last stages of this deficiency showed a reduction which in many cases was so small as to be within the experimental error of the technique employed, and in the most marked cases did not represent more than a 20 per cent. reduction. It is now claimed by Cramer, Drew and Mottram (1923) that the optical system employed by us for the counting of platelets was defective, and that owing to this the true nature of certain bodies or "protoplasmic *débris*," which, according to these workers, appear in the blood of vitamin A deficient rats, has been overlooked, leading to their inclusion by us in the platelet-count. They produce figures in support of this contention. (The claim that bacteria or lipoid globules could be mistaken for platelets is not worthy of consideration.) Objection is taken to the employment of an Abbé condenser in our optical system, which, as they correctly point out, reduces the resolving power of the objective; in their system the condenser is of the oil-immersion type with a working N.A. of 1·4. We were fully aware of the defects of the Abbé condenser before reading their communication, but surely such refinements in optical technique are hardly

necessary when dealing with objects, the average diameter of which is in the neighbourhood of 3μ. However, the issue is a perfectly straight one: Does the blood of a vitamin A deficient rat contain these protoplasmic *débris*—what one might term pseudo-platelets? If so are they present in such numbers as to explain the divergence in findings already referred to? It is hardly necessary to say that before publishing our findings, to which Cramer, Drew and Mottram take exception, this point had engaged our attention. A careful morphological study of the blood of deficient rats was made in the hope of finding an explanation of the differences in findings. None was forthcoming. These observations have been repeated in greater detail and form the subject of this communication.

PARALLEL PLATELET COUNTS MADE WITH THE TWO OPTICAL SYSTEMS.

The blood of normal and deficient rats was collected from the tail by the technique described by Cramer. The diluent employed was 2 per cent. sodium citrate in normal saline. Drops of this citrated blood were mounted between slide and coverslip, ringed with vaseline, and each preparation counted with the two optical systems. These two optical systems had the following composition:

TABLE I.

System.	Objective.	Ocular.	Condenser.
A.	Leitz 2 mm. apochromat. N.A. 1·4	Koristka 8, compensating	Zeiss oil-immersion, N.A. 1·4.
B.	Koristka 1/15″ semi-apochromat. N.A. 1·3	,,	Abbé.

At the same time drops of the citrated blood were spread on slides as ordinary blood smears, fixed in methyl alcohol and stained by Giemsa.

These stained preparations were examined for the presence of abnormal bodies similar in size to platelets, and in two instances the red cell : platelet ratio was estimated as well.

The results are tabulated below:

TABLE II.

Animal.	Red cell : platelet ratio. Op. system A.	Op. system B.	Stained smear.	Remarks.
Normal rat	8·4	8·6	—	—
—A rat 3848	10·4	10·9	10·9; no abnormal forms seen	Well-marked eye change in both eyes.
—A rat 3713	11·3	12·0	No abnormal forms seen	Well-marked eye change.
—A rat 3858	9·8	9·4	9·7; no abnormal forms seen	Commencing eye change in both eyes.

It will be seen that the counts obtained with the two optical systems show little or no difference, and the fact that the elements counted by us in the unstained preparations were really platelets is borne out by the counts made on the fixed and stained blood, where no doubt can exist as to what is and what is not a platelet.

COMPARISON OF DILUTING FLUIDS.

As the diluent used by Cramer, Drew and Mottram was Toisson's fluid, it was necessary to see whether the blood of deficient rats collected in this diluent gave the same counts as those obtained with the citrate-saline solution which we employ. The results of this experiment given in Table III show that the two diluents are equally good.

TABLE III.

Animal.	Red cell : platelet ratio.		Remarks.
	Toisson's fluid.	2% citrate in saline.	
—A rat 3859	9·7	10·4	No eye change. Weight stationary for 16 days.

MORPHOLOGICAL STUDY OF THE BLOOD OF DEFICIENT RATS.

In view of the findings given above it seemed hardly necessary to proceed further with this inquiry. However, this morphological examination was carried out anew on the blood of the deficient rats under investigation. Blood smears were made in the usual manner, and smears were prepared from citrated blood collected from the tail as for platelets counts. The diluent employed was either the ordinary citrate-saline, or with the addition of a fixative such as formalin (0·5 per cent.) or osmic acid (0·1 per cent.). The addition of a fixative to the diluting fluid is an advantage, and has been made use of by us in counting platelets for some time. The blood elements are fixed as they enter the anti-coagulant and their preservation is therefore ensured. Formalin has this defect, however, as a platelet fixative—it does not preserve the granules well. In this respect osmic acid is vastly superior. These preparations were stained either by Giemsa after fixation with methyl alcohol, or by the supra-vital method of Sabrazes (1923). This latter method consists in placing a drop of carbol-toluidine blue on the dried but unfixed smear, covering with a cover-slip and examining immediately. The smear should be freshly made. By this method the platelets stain a bluish-grey, their granules taking on a deep purple-black colour. As pointed out by Sabrazes, this is undoubtedly the simplest and most efficient method for the demonstration of the platelet granule, and incidentally the azurophil granules of the lymphocyte and large mononuclear leucocyte. The result of this morphological study of the blood of vitamin A deficient rats simply went to confirm our previous findings. No evidence whatever was obtained of the occurrence of protoplasmic fragments in this condition, the only elements observed being the red cells, leucocytes and platelets.

THE EFFECT OF ANTI-PLATELET SERUM IN DEFICIENT RATS.

In order to make this investigation as complete as possible it was decided to examine the effect of anti-platelet serum on the blood of vitamin A deficient rats. This serum contains an antibody specific for platelets, and it has been noted that its dosage can be so graded that the effect *in vivo* is limited to a reduction in platelets amounting to almost complete disappearance from the circulation, without the production of hæmorrhages and therefore without any disturbance in the red count. An anti-rat platelet serum was prepared in rabbits with platelets which were free from red cell contamination on microscopical examination. This serum was titrated in normal rats of from 70–80 grm. weight, and was found to produce almost complete disappearance of platelets from the circulation in 24 hours when inoculated intraperitoneally in a dose of 0·15 c.c. No purpura resulted from such a dose with the exception of an occasional skin hæmorrhage. Two rats suffering from a vitamin A deficiency were inoculated intraperitoneally with the above dose of anti-platelet serum, and the changes noted in the blood are tabulated below:

TABLE IV.

Animal.	Time of observation.	Weight.	Red cells.	R/P.	Platelets.	Leucocytes.	P.M. findings.
—A rat 3985	Before inoculation	69 gms.	8,280,000	8·5	975,000	10,660	—
	7.8.23 2.30 P.M.	0·15 c.c. anti-platelet serum inoculated intra-peritoneally.					
	8.8.23 10.30 A.M.	—	8,320,000	151·7	54,000	20,200	Some small petechiæ in abdominal wall at site of inoculation. One skin hæmorrhage seen. Otherwise normal.
—A rat 3989	Before inoculation	76 gms.	7,440,000	10·0	744,000	8,400	—
	7.8.23 2.30 P.M.	0·15 c.c. anti-platelet serum inoculated intra-peritoneally.					
	8.8.23 11.30 A.M.	—	7,080,000	356·3	20,000	20,200	No hæmorrhages in skin or subcutaneous tissue. Peritoneal cavity contains a small quantity of blood-stained fluid. Otherwise normal.

It will be seen that in these two rats, as in normal rats of approximately the same weight, the result of the inoculation has been the almost complete

disappearance of platelets from the circulation. The red cells are unaltered in number, whilst the leucocytes have undergone a definite increase in both cases. If the blood of these rats had contained "pseudo-platelets" one would have expected these elements to have remained unaffected, only the true platelets disappearing from the circulation. This was not the case; those elements counted by us as platelets had almost entirely disappeared. Then, again, if the true platelet content of the blood of these deficient animals had been only 200,000 or 300,000, one would have expected that this dose of serum would have produced a greater effect in them than it did in the normal rats. The degree of purpura was precisely similar in both categories of animals, *i. e.* none at all, or else a rare skin hæmorrhage.

DISCUSSION.

From the above findings there can be no doubt that the elements counted by us in deficient rats were in reality platelets, and that the difference between the optical system used by us and the one employed by Cramer, Drew and Mottram was not responsible for the divergence of findings. This is what one would have expected, for their claim did not really bear a close scrutiny. An analysis of their series of parallel counts, made on the blood of deficient rats with the two optical systems, reveals the fact that as the number of platelets decreases the protoplasmic fragments increase in number, the sum of the two remaining more or less constant. This alone should have made them suspect their explanation. Then, again, no mention is made in their communication of any examination of stained preparations of the blood of deficient rats. Had these authors seen these "protoplasmic *débris*" in fixed and stained preparations? What were their staining reactions? What form did these fragments present in fixed preparations? In the absence of any data on these points one is forced to conclude that these workers based their claim chiefly, if not entirely, on the examination of the unstained fresh blood only. Surely this was rather slender evidence from which to draw their conclusions, and hardly warrants the attack which they made on our technique! What, then, is the explanation of this discrepancy in findings? We would make the following suggestions: Contrary to the opinion expressed by Cramer, Drew and Mottram we find that the deficient rat bleeds with difficulty, and this becomes more and more marked as the deficiency becomes more pronounced; in fact, in some instances we have been unable to procure a reliable preparation from the tail, and have had to resort to cardiac puncture. It is an axiom in platelet counting that the blood must flow readily into the anticoagulant, otherwise platelets are lost or else partially altered by commencing coagulation and distorted in shape. Is it not possible that the "protoplasmic *débris*" of Cramer, Drew and Mottram are nothing more than altered and distorted platelets?

CONCLUSIONS.

(1) The optical system employed by us for the counting of platelets was not defective inasmuch as it was capable of performing the work it was called upon to do.

(2) We have been unable to confirm the observation of Cramer, Drew and Mottram as to the presence of "protoplasmic *débris*" in the blood of rats suffering from a vitamin A deficiency.

(3) The elements counted by us as platelets in the blood of such rats were in reality true platelets.

(4) We repeat that we are unable to confirm the original observation of Cramer, Drew and Mottram that vitamin A deficiency in the rat is productive of a severe thrombopœnia.

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THE INFLUENCE OF INSULIN ON THE FORMATION OF GLYCOGEN.

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An attempt was made, in the following experiments, to solve the question of the fate of the sugar disappearing from the blood after administration of insulin.

METHODS.

The experiments were performed on rabbits, the usual food of which consisted of cabbage and oats; in some cases the glycogen in the tissues was increased by a diet of carrots, sugar and oats. In one series of experiments, in order to get the liver and muscles free from glycogen, hypodermic injections of adrenaline were given the day before (Drummond and Paton, 1904; Agadschanianz, 1907). The animal received, per kilo weight, 1 c.c. of 1 : 1000 adrenaline solution (Parke, Davis & Co.), diluted to 2 c.c. with distilled water. In the earlier experiments I injected larger doses—from 1·5 to 2 c.c. adrenaline per kilo.

The blood-sugar was determined by Maclean's (1919) 1 c.c. method. The glycogen was estimated by Pflüger's method in 10 gm. of liver (pieces of liver

* The results of this investigation have been reported in a meeting of the Physiological Society, May 19th, 1923.

were taken from different parts of the organ), in 50 gm. skeletal muscles (different muscles of hind legs), and in the whole heart. The sugar, obtained by hydrolysis of the glycogen, was estimated by Bertrand's method.

GLYCOGEN CONTENT OF THE LIVER AND MUSCLES AFTER ADMINISTRATION OF INSULIN.

Macleod and his co-workers have observed in depancreatised dogs under the influence of insulin, a storage of glycogen in the liver (Banting *et al.*, 1922). I have repeated these experiments, but on normal rabbits.

From Table I we see that the glycogen content of the liver and muscles in very well nourished rabbits (fed on carrots, sugar and oats) after administration

TABLE I. INFLUENCE OF INSULIN ON THE GLYCOGEN CONTENT OF LIVER AND SKELETAL MUSCLES

RABBIT	DATE	DIET	WEIGHT IN gm	INSULIN INJECTED	PER CENT. OF INITIAL BLOOD-SUGAR	LOWEST PER CENT. OF BLOOD-SUGAR	PER CENT OF GLYCOGEN	
							LIVER	MUSCLES
II	28TH FEB	CARROTS & SUGAR AFTER ADRENALINE	1822	—	—	—	4·34	0·44
XII	13TH APRIL	CARROTS SUGAR AND OATS	2920	—	—	—	11·12	0·85
VI	15TH MARCH	CARROTS AND SUGAR	1835	0·02 gm CRUDE INSULIN	0·112	0·02 IN 2 hrs 45 mins	1·90	0·14
XVI	14TH MAY	CARROTS, SUGAR AND OATS	3168	0·007 gm INSULIN HYDROCHLORIDE	0·135	0·03 IN 2 hrs	2·87	0·09

NOTE. RABBIT II ON THE 26TH FEB. RECEIVED AT 3 P.M. AN INJECTION OF 1·5 CC. OF ADRENALIN + 1·5 CC OF DISTILLED WATER, AT 6 P.M. FOLLOWED THE SECOND INJECTION OF 1·0 CC OF ADRENALIN + 1·0 CC OF WATER ON 27TH FEB. FROM 12 A.M FEEDING WITH CARROTS AND SUGAR, KILLED ON 28TH FEB. AT 12·15 P.M.. RABBIT VI WAS KILLED 4½ HOURS AFTER ADMINISTRATION OF INSULIN; RABBIT XVI 5 HOURS AFTER. THESE TWO RABBITS AND RABBIT XII WERE NOT TREATED WITH ADRENALIN BEFORE FEEDING WITH CARROTS AND SUGAR.

of insulin is markedly lowered as compared with animals fed in the same way but without injection of insulin. In no cases have convulsions been observed, although the sugar content of the blood fell distinctly.

The disappearance of glycogen from both liver and skeletal muscles in animals which are given a dose of insulin sufficient to cause hypoglycæmic convulsions was observed independently by Dudley and Marrian (1923).

INFLUENCE OF INSULIN IN CASES OF ARTIFICIAL GLYCOSURIA.

I tried to obtain in rabbits a very high artificial hyperglycæmia, and to define (*a*) the influence of insulin on it, and (*b*) the influence of insulin on the storage of glycogen.

Influence of Insulin on the Blood-sugar.

In fasting rabbits of 2–3 kilos injected the day before with adrenaline, I introduced subcutaneously in control experiments large doses (20–30 gm.) of

glucose. The concentration of the solution employed varied from 10 to 13 per cent. It was injected during the first 2–3 hours, the experiment lasting 4–5 hours.

In the main experiments the procedure was the same except that glucose

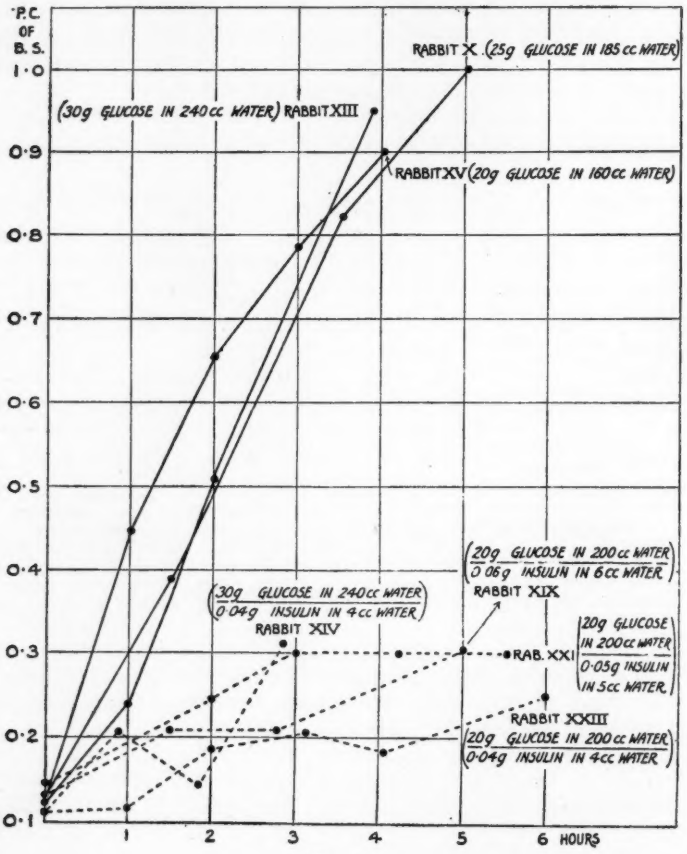

FIG. 1.

and insulin were injected subcutaneously also in large doses (from 40 to 60 mgm. crude insulin, 10 to 20 mgm. per injection).

All experiments are represented in the form of curves in Fig. 1. It will be seen that insulin always lowers the sugar content of the blood. If glucose is introduced alone, the blood-sugar in a few hours reaches nearly 1 per cent. If insulin is administered simultaneously with glucose, the percentage of blood-sugar does not exceed 0·3 per cent.

Condition of animals.—I always used the insulin (crude or hydrochloride) prepared in the Physiological Institute, University College, London. Testing this preparation (nearly 40 batches) on normal rabbits I often observed convulsions of two kinds: (1) flexion and extension of the whole body with final opisthotonos, and (2) more rarely, rotation of the animal round the longitudinal axis of the body. Neither of these convulsive movements

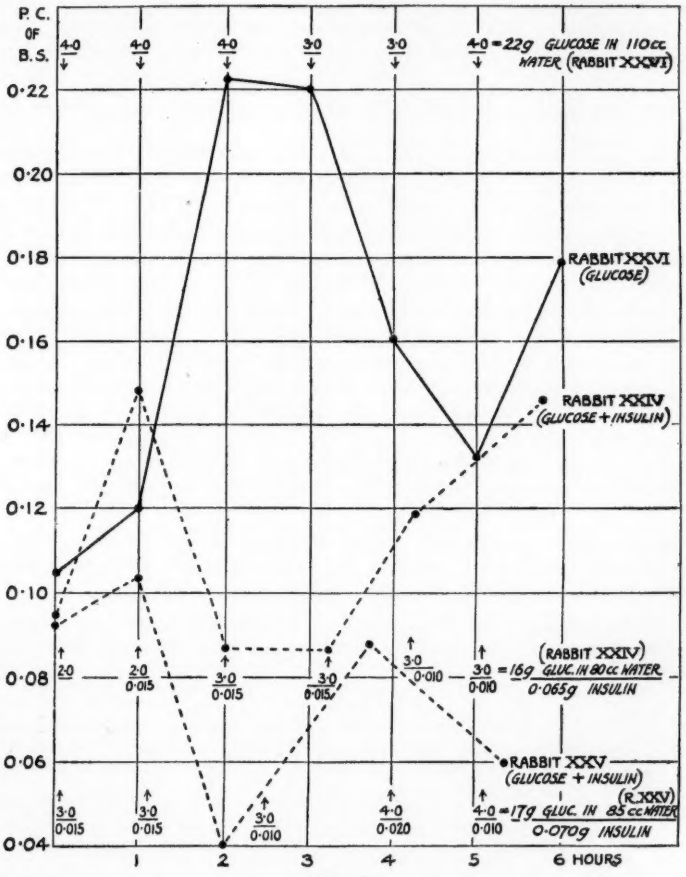

FIG. 2.

could be observed in the rabbits which received simultaneously glucose and insulin, the amount of insulin being 4 to 5 times greater than in the ordinary test.

In a few experiments the condition of the animals was nearly normal. In other cases, general apathy, weakness and even transitory paralysis of hind legs (in one case) were noticed. This result was not unexpected. The influence of a large quantity of glucose is opposite to that of insulin. Instead of excitement produced by insulin, depression is observed in cases of overloading the blood or the tissues with glucose. The animal is weak, his head drops, he shuts his eyes, his body rocks and he responds feebly to stimulation.

Introduction of glucose and insulin intravenously—In *post-mortem* examination of rabbits which received hypodermic injections of the solution of glucose or glucose and insulin, I observed that a large quantity of fluid remained unabsorbed under the skin. It is possible that insulin creates a condition which may influence the absorption of sugar solution. I repeated these experiments by introducing glucose and insulin intravenously. The solutions were introduced into the vein of one ear; from the vein of the opposite ear samples of blood were taken for blood-sugar estimation. No previous injection of adrenaline was made. The insulin used in these experiments was the same very active preparation as that employed in Experiments XXI and XXIII (see Fig. 1).

The results are represented in Fig 2. The top curve (the unbroken line) shows the blood-sugar content of a rabbit which received an intravenous injection of 2 per cent. glucose solution only (rabbit XXVI). The arrows and figures over it indicate the time and quantity of glucose injected. The two broken line curves are corresponding blood-sugar curves after administration of both glucose and insulin (rabbits XXIV and XXV). The arrows and figures under corresponding curves have the same meaning as the top figures

In both cases insulin lowered the content of blood-sugar. In rabbit XXIV the effect of insulin is not so pronounced as in rabbit XXV. One of the possible causes is the difference in the performance of the experiments: rabbit XXIV received during the first hour an injection of glucose only, rabbit XXV received at once both glucose and insulin. But of course there may be some other unknown reasons for the variable action of insulin, as has been already pointed out by some investigators (McCormick, Macleod, Noble and O'Brien, 1923).

The blood-sugar content after administration of the glucose solution only is not very high (maximum content 0·225 per cent.) This may possibly be due to the dilution of the blood by the solution.

I have only made two observations on the sugar content of the urine. In rabbit XXIV (glucose injected at the beginning of the first hour, insulin at the beginning of the second) and in rabbit XXVI (glucose only) I obtained urine from the bladder after the death of the animals. The estimation of sugar (Benedict's method) gave the following results:

Rabbit XXIV, 29 c.c. of urine with 2·2 per cent. of glucose, or 0·63 gm. glucose secreted.

Rabbit XXVI, 61 c.c. of urine with 2·0 per cent. of glucose, or 1·83 gm. glucose secreted.

It is not possible from these observations to judge the changes of the threshold for glucose in the kidney under the influence of insulin, but they indicate a way in which it is possible to examine these changes.

Influence of Insulin on the Storage of Glycogen.

The question of the storage of glycogen under the influence of insulin in cases of artificial hyperglycæmia is more complicated. The reason is that the glycogen content of the liver and muscles of rabbits, even under uniform conditions to which the animal is submitted, fluctuates. If large doses of adrenaline (1 c.c. or more of 1 : 1000 solution per kilo) are administered the day before, the liver and muscles are practically free from glycogen, but the action of the adrenaline has probably not quite passed off by the next day, when the experiment is performed. This is obvious from the comparatively high blood-

sugar content which was noticed in some cases. Moreover, these doses influence the weight of the animal and its capacity to store glycogen, as we shall see later, much more than moderate doses.

On the other hand, smaller doses of adrenaline do not render the liver and muscles quite free from glycogen. But even when the animals were not treated with adrenalin and were fasting before the experiment, the glycogen content of the liver and muscles varied very considerably. All these data are represented in Table II.

We must acknowledge that in this respect the rabbit is not a very suitable experimental subject. Its stomach always contains an unknown quantity of

TABLE II. CONTROL EXPERIMENTS.

RABBIT	DATE	WEIGHT IN gm		PER CENT LOSS OF WEIGHT	QUANTITY OF ADRENALINE INJECTED IN CC	PER CENT OF GLYCOGEN			TOTAL WEIGHT OF LIVER	QUANTITY OF GLYCOGEN IN LIVER IN gm.
		DAY BEFORE EXPERIMENT	DAY OF EXPERIMENT			LIVER	MUSCLES	HEART		
I	20TH FEB	863	803	6·9	2·0	NO	NO	–	–	–
V	9TH MARCH	1963	1792	8·7	4·0	NO	NO	–	–	–
IX	11TH APRIL	2264	2116	6·5	3·0	NO	NO	–	99	0
XVII	14TH MAY	2828	2711	4·1	2·0	2·96	0·15	0·33	111	3·28
XX	15TH JUNE	–	2468	–	NO	0·28	0·13	0·22	67	0·18
XXII	22ND JUNE	1575	1520	3·4	NO	3·84	0·25	–	58·5	2·24

food: the tissue cells are always ready to receive an indefinite quantity of the material for building glycogen. Thus, only very pronounced alterations in glycogen content under experimental conditions could be taken into consideration.

In Table III are represented the experiments with glucose alone or glucose and insulin together. These data do not give us evidence of a more pronounced accumulation of glycogen in the liver and skeletal muscles of rabbits which received glucose along with insulin than in those injected with glucose only, and, even if we assume that a certain storage of glycogen, as compared with control experiments (Table II), occurs, we are able to explain the disappearance from the blood of only a very limited part of the sugar.

The glycogen content of the heart (Tables II and III) undergoes variation which cannot be attributed to the action of insulin (*cf.* Dudley and Marrian, 1923).

Thus these experiments give evidence that insulin does not stimulate appreciably the formation of glycogen in normal animals.

I am much indebted to Prof. E. H. Starling for constant interest and advice during this work.

I wish to express my sincere thanks to Mr. R. K. Cannan for valuable help during the course of these experiments.

TABLE III INJECTION OF GLUCOSE AND INSULIN

RABBIT	DATE	WEIGHT		PER CENT. LOSS OF WEIGHT	QUANTITY OF ADRENALINE INJECTED IN C.C.	QUANTITY OF GLUCOSE INJECTED IN gm	QUANTITY OF INSULIN INJECTED IN gm	PER CENT OF GLYCOGEN			TOTAL WEIGHT OF LIVER IN gm	QUANTITY OF GLYCOGEN IN LIVER IN gm
		DAY BEFORE EXPERIMENT	DAY OF EXPERIMENT					LIVER	MUSCLES	HEART		
IX	22ND MARCH	3038	2649	12·8	3·0	35·0	0·03	0 46	NO	–	99	0·45
XIV	25TH APRIL	2529	2247	11·1	2·5	30·0	0 04	3·07	0·03	0·52	89·5	2·74
XIX	30TH APRIL	2151	1903	11·5	2·5	20 0	0·06	2·36	0·11	0·34	58	1·36
XXI	15TH JUNE	–	3486	–	NO	20·0	0·05	3·33	0·09	0·35	75	2·49
XXIII	22ND JUNE	1971	1878	4·7	NO	20·0	0·04	5·56	0·15	0·28	67	3·72
XXIV	25TH JUNE	–	3269	–	NO	16·0*	0·065*	1·48	0·29	–	102	1·50
XXV	4TH JULY	2859	2728	4·5	NO	17·0*	0·07*	3·05	0·25	–	89	2·71
INJECTION OF GLUCOSE												
X	28TH MARCH	2051	1946	5·1	2·0	25·0	–	3·63	0·06	–	72	2·61
XIII	19TH APRIL	2365	2163	8·5	3·0	30·0	–	0·92	TRACES	0·55	64	0·58
XV	8TH MAY	2969	2712	8·6	3·0	20·0	–	0·17	TRACES	0·50	82	0·14
XVIII	29TH MAY	1970	1733	12·0	2·0	20·0	–	1·11	0·06	–	75	0·83
XVI	13TH JULY	2465	2340	5·1	NO	22·0*	–	5·00	0·35	–	98	4·9

* INJECTED INTRAVENOUSLY

The expenses of this research were borne by the Medical Research Council.

SUMMARY.

1. The glycogen content of the liver and skeletal muscles in very well-nourished rabbits, after administration of insulin, is markedly lowered as compared with animals fed in the same way but without the injection of insulin.

2. Insulin always lowers the sugar content of the blood even after subcutaneous or intravenous injection of very large doses of glucose (25–30 gm. in a rabbit of 2–3 kilos).

3. There is no pronounced accumulation of glycogen in the liver and skeletal muscles in rabbits which have received glucose and insulin simultaneously.

4. Insulin has apparently no influence on the glycogen content of the heart.

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COMPLEMENT-FIXATION IN STREPTOCOCCAL INFECTIONS.

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THE work to be recorded was undertaken to see how far the complement-fixation test is successful for identifying streptococcal infections. Fixation of complement during union of antigen and specific antibody is the basis of a delicate test for the presence of specific antibodies in the blood, and the presence of these is regarded as important evidence of specific infection.

It was considered that if successful in the case of streptococci, the test might prove to be of great value in the elucidation of the ætiology of rheumatic fever. At present the precise part played by streptococci in rheumatic fever is a matter of controversy. It seemed possible that by employing this serological procedure, one might, for instance, be able to obtain information as to the pathogenic significance of individual strains of streptococci isolated from the patient.

There was also the possibility that the reaction might throw welcome light on certain other conditions of obscure or uncertain ætiology, where a streptococcus has been suspected of causal agency; for instance, that it might help to differentiate between the various types of chronic arthritis.

With these ends in view preliminary observations were made, first to endeavour to see if complement-fixing antibodies are constantly present in streptococcal infections; secondly, to investigate the degree of specificity of these antibodies.

Attention was first given to the results by the complement-fixation test in rabbits prepared with streptococci of known character. During these experiments a routine technique of quantitative character was worked out, and the degree of specificity of the test for differentiating between the various races of streptococci determined. Naturally occurring infection in human beings with streptococci, the characters of which had been definitely ascertained, were studied next. Guided by the results of these preliminary experiments, an attempt was made to find the best method for applying the test to the study of rheumatic fever and allied diseases.

REVIEW OF THE LITERATURE.

There is evidence from previous work that complement-fixing antibodies are present in many streptococcal infections. They have been demonstrated in

the serum in cases of scarlet fever (Foix and Mallein, 1907; Schleissner, 1909; Castex, 1909), erysipelas, streptococcal abscess, puerperal fever, streptococcal pleurisy, streptococcal arthritis (Castex, 1909), and "endocarditis lenta" (Major, 1912; Kinsella, 1917). Aschner (1917) found that the serum of a patient suffering from subacute streptococcal endocarditis gave marked complement-fixation with an antigen prepared from the homologous coccus; with a polyvalent antigen prepared from organisms isolated from other cases of malignant endocarditis a positive but less marked fixation occurred.

Attempts to classify the streptococcus group by the complement-fixation test have not been successful. Kinsella and Swift (1917) and Katherine Howell (1918) could not harmonise a grouping by this method with any other classification.

Marked specificity among streptococci has been observed by Floyd and Wolbach (1913–1914) using the complement-fixation method.

The test has been applied to the study of chronic arthritis by Richards (1920). He obtained positive reactions in 68 out of 104 cases, using as antigens mixtures of the strains of *Streptococcus viridans* isolated from the infective foci.

TECHNIQUE.

The reaction was made quantitative by testing the serum in falling dilutions. Details of the technique adopted are summarised as follows:

1. *Hæmolytic system.*—This was adjusted by complement titration, determining the minimum amount of guinea-pig's serum which produces complete hæmolysis of 0·1 c.c. of a 5 per cent. saline suspension of sheep's red corpuscles in presence of the unit of hæmolytic amboceptor and saline to 1 c.c. when incubated in the water-bath at 37° C. for ½ hour.

The unit of hæmolytic amboceptor is twice the minimal dose which completely hæmolyses the unit of sheep's cells in presence of 0·1 c.c. of guinea-pig's serum diluted 1 in 10 and saline to 1 c.c. when incubated in the water-bath at 37° C. for ½ hour.

The unit of sensitised corpuscles to be used in the test as an indicator for complement is thus 0·1 c.c. 5 per cent. suspension of corpuscles *plus* 0·1 c.c. diluted hæmolytic serum containing 2 M.H.D. amboceptor.

The unit of complement used in the test is 0·1 c.c. of diluted guinea-pig's serum containing 1½ or 2 M.H.D. of complement.

2. *Antigen.*—Various preparations of the organism were investigated to determine whether the delicacy of the reaction can be influenced in this way. Very little difference was found between suspensions of the organism grown in different media. A saline suspension of the coccus was slightly more delicate as an antigen if clumps of cocci were removed by light centrifuging. Autolysates, aqueous extract, NaOH extract, the acetone extracted coccus, and this preparation trypsinised for various times, were investigated. None of these gave a more delicate reaction than a simple saline suspension of the coccus. This preparation was used in the experiments to be recorded; the suspension was heated to 60° C. for one hour, and 0·5 per cent. phenol added. Before each test the anti-complementary power of the antigen is determined. Units of complement are incubated at 37° C. for one hour with falling quantities of antigen—viz. 0·1 c.c. doses containing respectively 400, 200, 100 and 50 million cocci. The unit of sensitised corpuscles is then added to each tube, and after further incubation for ½ hour the anti-complementary dose is known. When this dose is one which causes a trace only of inhibition of hæmolysis, half the quantity is used in the test; in other cases one quarter the anti-complementary dose is used.

3. *The test.*—Falling amounts of inactivated serum—1/100 c.c., 1/200 c.c., 1/400 c.c., etc.—are incubated at 37° C. for 1 hour with 1½ or 2 M.H.D. of complement, and ½ or ¼ the anti-complementary dose of antigen. More precisely each tube contains 0·1 c.c. diluted guinea-pig's serum containing 1½ or 2 M.H.D. of complement, 0·1 c.c. requisite dilution of

antigen, and 0·1 c.c. serum diluted 1 in 10, 1 in 20, 1 in 40, etc. In addition the following control tubes are put up:

1. Serum control:
   0·1 c.c. serum diluted 1 in 10, 1 in 20, etc., each with the unit of complement.
2. Antigen control:
   The dose of antigen used in the test, with the unit of complement.

After this incubation the unit of sensitised corpuscles is added, and the total volume in each tube made up to 1 c.c. with saline. After a further incubation at 37° C. for ½ hour the results are read off. A range of serum dilutions sufficient to bring out the end-point is tested. The final dilution of serum in each tube is 1 in 100, 1 in 200, 1 in 400, etc., and the actual amount of serum present in each tube is 1/100 c.c., 1/200 c.c., etc.

OBSERVATIONS WITH SERA FROM ARTIFICIALLY IMMUNISED RABBITS.

Before investigating naturally occurring infections in the human being, it was desirable to determine the reaction of an animal when treated with a known type of streptococcus. The object of these experiments was to see if complement-fixing antibodies can be detected in the serum of such an artificially immunised animal, and to what extent they can be produced. Further, one wished to determine the behaviour as antigens of the three main groups of streptococcus—*pyogenes*, *salivarius* and *fæcalis*—particularly as regards the degree of specificity of the antibodies produced by typical members of each group.

Three rabbits were immunised by Dr. M. H. Gordon, one with *Streptococcus pyogenes*, Type I (Gordon, 1921), the second with a typical example of the *Streptococcus salivarius* group, the third with a typical example of the *Streptococcus fæcalis* group. After 12 days, during which the animals were given five injections, samples of serum were taken and tested. Injections were intravenous. The *pyogenes* animal received an initial dose of 250 millions, increased to 2000 millions in 12 days; initial doses for the *salivarius* and *fæcalis* animals were 500 millions, the dosage again being increased to 2000 millions in 12 days. Further samples were tested at intervals during a period of 2½ months, immunisation being continued over this time, the dose being increased in each case to 16,000 millions.

Each sample was tested with the three antigens under investigation; the result with normal rabbit serum and these antigens was negative in each case. The first samples of serum from the *salivarius* and *fæcalis* rabbits gave negative reactions with all three antigens, and by testing at intervals the increase in complement-fixing antibodies could be observed.

The information obtained from these experiments may be summarised as follows:

(1) Rabbits treated with typical examples of the *pyogenes*, *salivarius*, and *fæcalis* groups respond by production of complement-fixing antibodies in sufficient quantity to be readily demonstrated by the technique adopted.

(2) Complement-fixing antibodies appear to be more rapidly formed to *S. salivarius* and *S. fæcalis* than to *S. pyogenes*. The highest titre with the *salivarius* and *fæcalis* rabbits—1 in 800 and 1 in 3200 respectively—was obtained on the eighteenth day; the highest titre with the *pyogenes* rabbit—1 in 1600—was obtained after two months.

(3) *Pyogenes* serum shows little specificity for the homologous coccus. The serum was tested with various heterologous cocci of the *salivarius* and *fæcalis* groups, and in all cases fixation was obtained, in the majority to the same titre as that of the homologous reaction.

(4) A definite specificity for the homologous coccus is observable in both

salivarius and *fæcalis* sera; in both cross-fixation occurs with the examples of the other groups tested, but this is to a lower titre than the homologous fixation.

OBSERVATIONS IN HUMAN INFECTIONS.

(A) *Infection with Non-hæmolytic Streptococci.*

Malignant endocarditis affords a good example of infection with the non-hæmolytic group of streptococci. Ten examples of this infection were investigated; in each case the infecting coccus was isolated by blood-culture. Seven were *Streptococcus salivarius* infections, three *Streptococcus fæcalis*; all were of some months' duration.

The patient's serum was tested with the homologous coccus and with heterologous cocci (1) from the other cases investigated as they became available, (2) isolated previously by Dr. M. H. Gordon from similar cases. All these cocci were shown by Dr. Gordon to be serologically distinct when tested by the method of agglutination and absorption of agglutinin. Negative controls in each case were satisfactory, the reaction of these antigens with normal human serum being negative at 1 in 100.

In all cases the patient's serum gave a positive reaction with the homologous coccus. The end-point varied from 1 in 800 to 1 in 6400. The serum showed a high degree of specificity for the homologous coccus. No cross fixation at all was demonstrable with some of the heterologous cocci tried; with others cross-fixation occurred, both with cocci belonging to the same group as the homologous coccus and with examples of the other groups, though this cross-fixation was to a much lower titre than the homologous fixation.

A typical example of the results obtained in these cases is shown in the following table:

Case: Andrews. S. salivarius *isolated by Blood Culture.*

Antigens.		Andrews serum. 1 in—							
		100	200	400	800	1600	3200	4800	6400
Salivarius	Andrews	++	++	++	++	++	++	+	−
	Ross	++	++	+	+−	−			
	Day	+	+−	−	−	−			
	Hodson	+	+−	−	−	−			
	Magona	+	−	−	−	−			
Fæcalis	Bradshaw	+	+−	−	−	−			
	Harvey	+−	−	−	−	−			
Pyogenes Type 1	155	++	++	+−	−	−			

++ = Complete inhibition of hæmolysis.
\+ = Between complete inhibition and 50% inhibition.
+− = Between 50% inhibition and complete hæmolysis.
− = Complete hæmolysis.

It was observed in these cases that fixation with a heterologous coccus of the same group as the homologous coccus (*i. e. salivarius* or *fæcalis*) may be no greater or even less than the cross-fixation with a member of another group.

The maximum amount of complement fixed by 1/100 c.c. of serum from one of these cases—a *Streptococcus fæcalis* infection—was determined. The end-point with $1\frac{1}{2}$ M.H.D. was 1 in 1600; by testing with increasing doses of complement it was proved that 1/100 c.c. of serum fixed at least $3\frac{1}{2}$ M.H.D.

(Note: the sera of these cases of malignant endocarditis and the rabbits' sera discussed in the last section were tested by methods of agglutination and absorption of agglutinin by Dr. M. H. Gordon.)

(B) *Infection with Hæmolytic Streptococci.*

The results obtained in eight of these infections are recorded. All cases were tested with a stock *S. pyogenes*, Type I, and five were also tested with an antigen prepared from the actual infecting coccus. In each case a known *pyogenes* serum (rabbit) was put up as a control and was satisfactory. These observations indicate that the serum from a case of *S. pyogenes* infection is poor in complement-fixing antibodies—a finding not unexpected, as these infections are acute and of comparatively short duration.

Cases 1, 2 and 3.—Erysipelas. 12 days', 3 weeks', and 8 weeks' duration respectively. (Axillary abscess in case 3.) Reaction in each case negative at a serum dilution of 1 in 50.

Case 4.—Cellulitis. 4 weeks' duration. Reaction negative at 1 in 50.

Case 5.—Acute mastoiditis. 2 weeks' duration. Reaction with *S. pyogenes*, Type I, positive to 1 in 200. Antibodies disappeared with recovery.

Case 6.—Mastoid abscess. 17 days' duration. Reaction negative at 1 in 50.

Case 7.—Septicæmia. 10 weeks' duration. Reaction with infecting coccus positive to 1 in 400, with a representative of the *salivarius* group negative at 1 in 50, and with a typical *S. fæcalis*, positive to 1 in 100. The serum appears more specific than that of the artificially immunised rabbit.

Case 8.—Suppurative arthritis. 10 days' duration. Reaction positive to 1 in 50.

A CONSIDERATION OF THE POSSIBILITY OF IDENTIFYING STREPTOCOCCAL INFECTIONS BY THE COMPLEMENT-FIXATION TEST.

Dr. Gordon's (1922) observations with agglutinin have shown that while the hæmolytic streptococci comprise three serological types only, the non-hæmolytic group is divisible into extremely numerous types, which exhibit serologically (agglutinin) a high degree of specificity.

Results so far recorded suggest that in *salivarius* or *fæcalis* infections of some months' duration, complement-fixing antibodies to the infecting coccus would be demonstrable in the patient's serum. The fact that a positive reaction is not usually obtained in the acute conditions caused by *S. pyogenes*

does not lessen the possible value of the test; diagnosis in such cases is not usually a matter of difficulty.

Two possible methods suggest themselves for applying this test to the investigation of cases suspected on clinical grounds to be streptococcal infections.

First the use of polyvalent antigens may give evidence of streptococcal infection, though on account of the large number of highly specific types which make up the non-hæmolytic group, it is obvious that little value can be attached to a negative reaction. This procedure has been tried in a number of cases where, clinically, a streptococcus was suspected of causal agency, including cases diagnosed as infective endocarditis, with negative blood cultures, iritis, rheumatic fever, chronic arthritis. The antigens used, viz. *S. pyogenes*, Type I, polyvalent *S. salivarius*, and polyvalent *S. fæcalis*, have been tested with normal human sera, and shown to give negative reactions at a serum dilution of 1 in 50 in each case (seven normal sera were tested). The large number of negative results obtained served as further negative controls, and it would therefore appear probable that a well-marked positive reaction can be regarded as indicating streptococcal infection. But the method is unsatisfactory, because at present representative antigens cannot be made, and consequently infection cannot be excluded by a negative result.

Out of five cases diagnosed clinically as malignant endocarditis, but which gave negative blood cultures, only two gave a positive reaction, and in only one of these was the reaction well marked. Of twenty cases of chronic arthritis a well-marked positive reaction was obtained in seven, a doubtful reaction (weak positive) in seven, and a definitely negative reaction in six.

The second method is to test the patient's serum with individual strains of streptococci isolated from possible infective foci—a tooth-socket, a tonsil, or the intestinal tract; or in cases of rheumatic fever, with streptococci isolated from various organs and body-fluids either during life or *post mortem*.

The question of the *rôle* of streptococci in the ætiology of rheumatic fever remains unanswered. The view has been repeatedly expressed that rheumatic fever is a specific bacterial infection, and that the organism is a streptococcus of the non-hæmolytic group. It is hoped that further evidence as to the pathogenic significance of streptococci isolated from various organs and body-fluids in cases of rheumatic fever and chorea may be obtained by applying the complement-fixation test for the detection of specific antibodies in the patient's serum. The study of rheumatic fever along these lines is in the early stages; the number of cases and controls investigated is not sufficient for any definite statement to be made. A brief record, however, of the work carried out so far may perhaps be added. As the tonsil has been suspected of being the site of invasion, it seemed best to investigate, first, the problem as to whether or not a streptococcus giving a positive reaction with the patient's serum can be isolated from the tonsil. Cases were therefore studied in this way as opportunity offered; several strains of streptococci were isolated from tonsil cultures, and each strain tested with the patient's serum. Cultures were aërobic, and on trypagar enriched with blood or ascites fluid. Of seven cases so investigated, positive results were obtained in three:

(1) Rheumatic heart disease, subcutaneous nodules; duration 3 months.

Reaction of serum positive with 1 out of 7 streptococcus colonies isolated, a *S. pyogenes*, giving an end-point of 1 in 1600. (Agglutination test, by Dr. M. H. Gordon, positive to 1 in 200 with this coccus.)

(2) Rheumatic fever. Duration 6 weeks. Reaction positive with 1 out of 19 streptococcus colonies isolated; this organism gave the carbohydrate reactions of *S. salivarius*; end-point 1 in 400.

(3) Rheumatic fever. Duration 5 months. Reaction positive with 1 out of 18 colonies, this organism giving the carbohydrate reactions of *S. pyogenes*; end-point 1 in 200.

In the other four cases, including two of acute rheumatism, a streptococcus giving a positive reaction with the patient's serum could not be isolated from the tonsil.

The number of streptococcus strains isolated which gave a negative reaction is striking, and suggests the possibility that the cocci giving positive reactions are heterologous organisms having a saprophytic existence on the tonsil.

It is suggested that the complement-fixation test should prove of value in the experimental study of rheumatic fever and allied diseases.

SUMMARY.

(1) If the complement-fixation test is successful for identifying streptococcal infections, it might prove of value in the experimental study of rheumatic fever and allied diseases. The observations recorded were carried out to see how far the reaction is of value for this purpose.

(2) Rabbits respond to treatment with typical examples of the three main groups of streptococci by production of complement-fixing antibodies in sufficient quantities to be easily demonstrable. A definite specificity is observable in both *salivarius* and *fæcalis* sera, but a *pyogenes* serum shows little specificity for the homologous coccus.

(3) Ten cases of infective endocarditis were investigated. In all the patient's serum gave a positive reaction, to a high titre, with the homologous coccus (obtained by blood culture); a high degree of specificity is observable, though cross-fixation may occur with a streptococcus of any group. The groups *pyogenes*, *salivarius* and *fæcalis* are not distinguishable by complement-fixing antibodies.

(4) A positive reaction is not usually obtained in the acute conditions caused by *Streptococcus pyogenes*.

(5) Two methods of applying the test to the experimental study of rheumatic fever and allied diseases are suggested, viz.:

a. By using polyvalent antigens.

b. By testing the patient's serum with individual strains of streptococci, isolated from possible infective foci, blood, pericardial fluid, joint fluid, etc.

The second procedure appears, at present, to offer the best means of applying the test to obtain further evidence as to the *rôle* of streptococci in these diseases. It is suggested that, in cases of some months' duration, the complement-fixation test affords a reliable criterion as to the pathogenic significance of a non-hæmolytic streptococcus isolated from the patient.

The work recorded is part of a research on the streptococcus group which is being carried out for the Medical Research Council in the Pathological Department of St. Bartholomew's Hospital.

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SOME CULTURAL CHARACTERISTICS EXHIBITED BY SEROLOGICAL TYPES OF MENINGOCOCCI.

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As early as 1909 Arkwright found some evidence that different strains of meningococcus fall into various groups as regards their agglutination reactions. He also found differences in their power of fermentation of glucose and maltose. Martin (1910) discovered that some meningococcus strains ferment dextrose less rapidly than maltose, while Griffith (1916), on the basis of simple agglutination tests, divided 34 cerebro-spinal strains from an epidemic into two main groups which, with few exceptions, exhibited corresponding fermentative properties, namely, (1) strains which fermented maltose more than glucose, and (2) strains which fermented glucose more than maltose. In 1915, on the basis of agglutination tests, Gordon and Murray (1915) proposed the division of these organisms into four types. Opinion is divided as to the justification of this grouping, and many authors consider that there is so much affinity between their types I and III on the one hand, and II and IV on the other, that it is more rational to recognise two main types only. Continental workers

have always insisted on two broad groups—for example, the meningococcus and parameningococcus of the French writers. Hitherto no serious attempts have been made to correlate these types with cultural characteristics, or to associate the appearances of cultivations of different strains of this organism with any other definite grouping. Thus the following passage occurs in a paper by Nicolle, Debains, and Jouan (1918): "Considérés au point de vue de leurs 'caractères généraux' (morphologie, aspect des cultures, propriétés biologiques), les méningocoques offrent des traits communs, que chacun connaît aujourd'hui et des différences individuelles. Ces différences, légères et sans relation entre elles, ne permettent pas de créer des groupes distincts. Nous allons montrer que les méningococciques, envisagés au point de vue de leurs 'caractères antigènes' manifestent, par contre, des dissemblances marquées et concordantes, imposant l'idée de type tranchés."

Meningococcus agglutinating serum for four types is laborious to prepare, and as there is not a great demand for it, especially during the summer months, it is often difficult to obtain. Moreover, the accurate typing by agglutinating sera is an intricate process requiring considerable care. Many strains when first isolated are practically inagglutinable, and some, even after cultivation on artificial media for some time, are only agglutinated by a serum at several times its titre-strength, which has led not a few observers to class a strain as not belonging to any of Gordon's four types. The titres of the type sera must be accurately known, otherwise we may give undue weight to group agglutination, which sometimes occurs. When sera are employed in greater concentrations than their titre-strength we must be alive to the possible presence of "zone" effects which may completely invert the typing. To an experienced hand this presents no real difficulty, but it may frequently fall to the lot of one not specially versed in the complexities of agglutination to have to assign its type to a newly isolated strain. It would therefore be convenient to have a set of cultural tests, if possible, by which the types could be distinguished whenever a strain is isolated from the spinal fluid. By a modification of technique I have succeeded in obtaining quite marked cultural differences, especially in the case of the two main types, which agree remarkably well with the serological grouping.

METHOD OF EXPERIMENT.

While casting round for a medium which should at the same time be cheap and favourable for the growth of the meningococcus, Gordon decided upon one made by dissolving agar in trypsinised broth and adding a saline extract of peaflour. This is known as "trypagar" (trypsin-broth-pea-agar). This is the medium chiefly used in the present work. Its preparation is as follows:

Mince 1 lb. of meat and boil in 1 litre of water; filter, and make faintly alkaline to litmus; heat at 85° for five minutes and then cool to 37°; add 1 per cent. trypsin and keep at 37° for three hours; make slightly acid and boil for twenty minutes. After standing overnight syphon off the clear fluid; this is called "trypbroth." Take 1 litre of trypbroth and add 2 per cent. agar; dissolve and make slightly alkaline; cool, add egg for clearing, autoclave and filter; add 5 per cent. peaflour extract; steam on two successive days for twenty minutes.

The peaflour extract is made by grinding a 10 per cent. emulsion of peaflour in 10 per cent. NaCl in a mortar. It is then steamed for twenty minutes and filtered.

It was observed that on this medium some of the stock strains tended to die out sooner than others—a circumstance which suggested a thorough investigation to discover whether this feature could be made the basis of a method of typing meningococcus cultures.

Petri plates of about 9 cm. diameter were filled with trypagar to a depth of 7–9 mm. This is considerably more medium than is usually employed for plate cultures, but it is necessary for uniform results. In making these plates contamination occurs very readily and certain special precautions are necessary. Owing to the relatively large amount of medium in each plate, a good deal of condensation water forms on the lid when the plates get cold. This can be removed by carefully lifting the lid slightly and tilting, which causes the drops of water to run off at the side. If this is not attended to the sides of the plate remain very moist, and the water soon absorbs enough nutritive material from the medium to enable air organisms to grow round the edges. After pouring, the plates were placed in the incubator at 37° for a day or two to free the surface of the medium from loose water, and incidentally, also, to ensure their sterility. They were then turned upside down, and a circle whose diameter was 5·2 cm. was inscribed on the glass concentric with the plate. Six equidistant points were marked on this circle as the sites of the inoculations. In this way any slight irregularities in the plate that happened to be present were largely counteracted, as they mostly consist of concentric ripples, and a medium of uniform thickness was obtained. The two strains of meningococcus to be tested were inoculated from overnight cultures alternately by just touching the six points with the charged platinum needle. The appearance of the plate is shown in Figs. 2 and 3. They were then incubated upside down at 37°. After they had ceased growing—usually in 6 or 7 days—small samples were taken from the edge of the colony from day to day and inoculated on trypagar slopes to see whether the colony was still viable. A fresh bit of the edge was used each time, because it was necessary to avoid any portions which may have been pushed further out into the medium on a previous occasion and thus have been enabled to grow afresh, with, consequently, an extended lease of life. It was found on trial that the centre of the colony did not live longer than the edge; in fact it usually died first. It was essential in the first instance to see how different strains of the same serological type behaved.

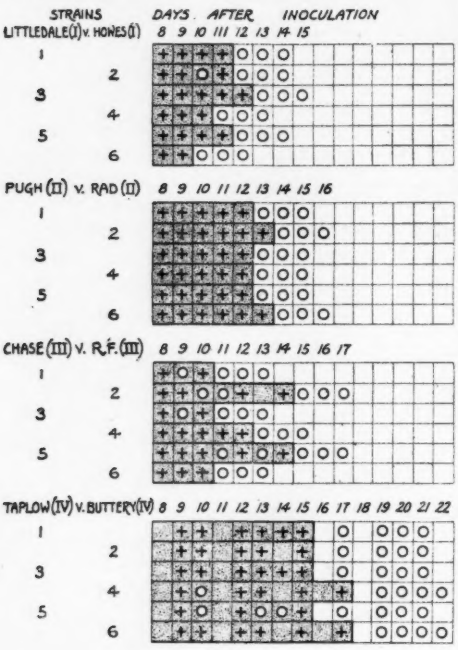
The strains used in this work, numbering about 30, came from several different countries, and although not large in number they may be taken as fairly representative of the disease in various parts of the world. They were all spinal in origin. Some of them were Gordon's original strains as received from the Royal Army Medical College; others were obtained from other parts of Great Britain, Denmark, Poland, South Africa and the United States. They had been cultivated on artificial media for variable periods ranging from the early part of the war up to the present time. There is no evidence that any of them have changed their serological type since isolation. Most of them have been tested several times.

VIABILITY TESTS.

In Table I two strains* of each type are compared with one another as regards length of life on trypagar plates. As stated above, alternate colonies are from the same strain. It will be noticed that the variations which occur are about as great between colonies of the same strain as between different strains of the same type. These results are quite representative of many tests that have been made. Occasionally anomalous results have been obtained where one or two colonies have long outlived the rest owing to the medium having separated from the opposite side of the plate and shrunk on account of drying, but where no unusual happening of this nature has taken place the results have been quite uniform. The widest variations are 3 or 4 days. Sometimes the sample taken on a certain day fails to grow, although a subsequent sample shows that the whole of the colony is not dead. This usually presages the end. Several negative tests had to be obtained before finally concluding that the colony was dead. Where a blank space is left the colony was not tested on the day in question.

TABLE I.

COMPARISON OF THE LENGTH OF LIFE OF DIFFERENT STRAINS OF THE SAME TYPE ON TRYPAGAR.


STRAINS
DAYS AFTER INOCULATION
LITTLEDALE(I) v. HOWES(I)
8 9 10 11 12 13 14 15
1
2
3
4
5
6
PUGH (II) v. RAD (II)
8 9 10 11 12 13 14 15 16
CHASE (III) v. R.F. (III)
8 9 10 11 12 13 14 15 16 17
TAPLOW (IV) v. BUTTERY (IV)
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Turning now to a comparison of strains of different types, Table II shows that there is a large difference between types I and II, the former usually dying out much earlier. The same distinction is found between types I and III, in favour of a considerable longer life for III. The difference between II and III is not nearly so marked, and it is doubtful whether any conclusion can be drawn as to any advantage in viability possessed by one of these types over the other. The same applies to types II and IV.

The last three experiments in Table II are anomalous and deal with two strains, Sp 38 and 46 B, of rather uncertain type. More will be said about these later.

It was interesting now to enquire whether the same distinction between types I and II obtained on a serum-agar medium. The basis of this medium

* In this paper each strain is referred to by name, and its type, in conformity with Gordon's grouping, is appended in brackets.

TABLE II

COMPARISON OF THE LENGTH OF LIFE OF DIFFERENT TYPES ON TRYPAGAR.

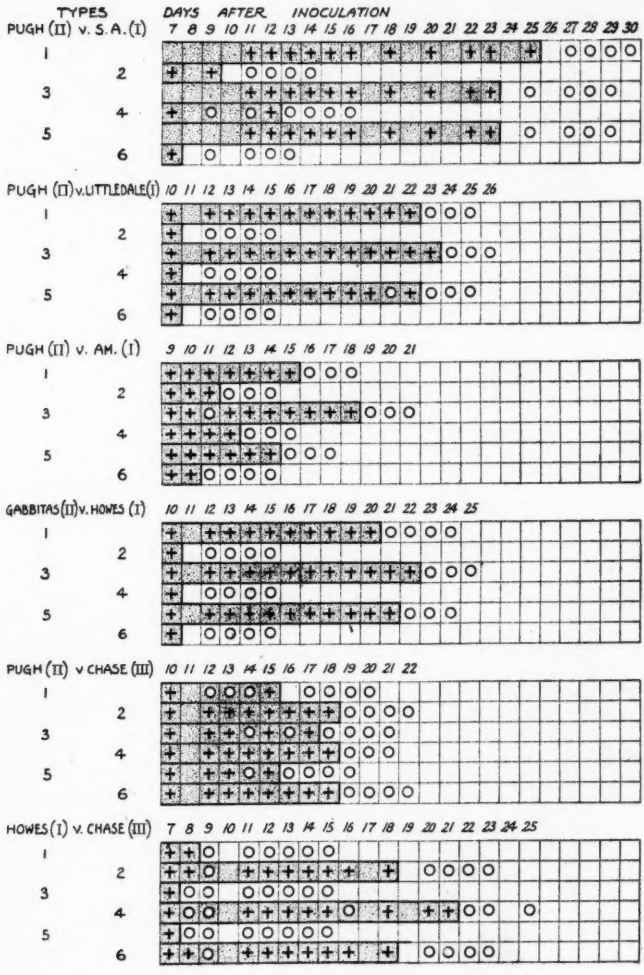
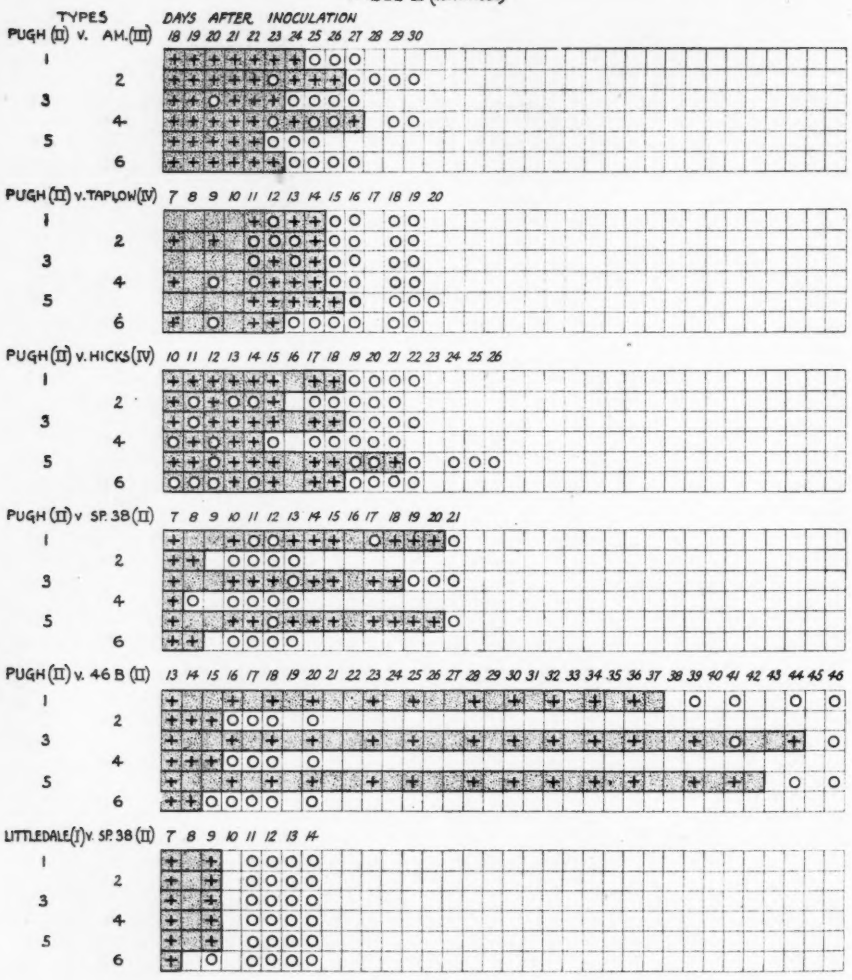


TABLE II (CONTINUED)

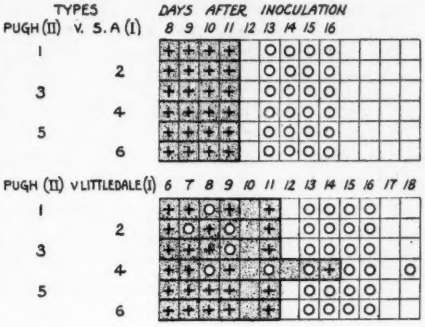


was trypsinised meat broth with an addition of horse-serum of about 1 in 4. Table III shows that there is practically no difference in the length of life of the two types mentioned on this medium, which proves, among other things, that it can hardly be different powers of resistance to drying *per se* that account for the differences on trypagar.

To sum up, these viability tests on trypagar show a clear distinction between type I and the other three types, in favour of a considerably shorter life for the former. In many cases it survives less than half the time that the others do. Between types II, III and IV there are no such clearly defined differences, and it is hardly possible to separate them by this test. The viability on a serum-agar medium, on the other hand, is about the same for types I and II. The relatively short life of type I on trypagar may be due to the formation of larger amounts of some decomposition product, possibly the alkali produced.

TABLE III.

COMPARISON OF THE LENGTH OF LIFE OF TYPES I & II ON SERUM AGAR.



Appearances of Colonies.

The cultural features now to be described were obtained on plates of trypagar similar to those employed above. By virtue of the thickness of the medium (7–9 mm.), the colonies were able to grow to a large size and develop characteristic surfaces which are not observed when growth is checked at an earlier stage. This seems to be the reason that they have not been described before, for although the medium is not new, the method of employing it in a much thicker layer in plates does not appear to have been tried previously.

The strains of type I again show the greatest individuality. This type grows as a whitish or pale yellowish colony with a more or less scalloped or indented border. From the beginning the surface is usually somewhat rough and may be intersected by furrows. About the time the colony attains its full size papillæ begin to appear in most cases, scattered all over the surface. Some are larger than others, but occasionally the entire surface is covered with *minute* papillæ. The time of their appearance varies a good deal, as it depends upon the rate of growth of the colony. They are presumably an expression of the reaction of the organism against one of its own metabolic products—perhaps the relatively strong alkalinity produced in the medium. About this time, or a little later, a halo begins to form round the colony which gradually extends outwards in the medium, often eventually taking the form of a ring surrounding the colony. The formation of this halo is probably caused by the precipitation of a salt of legumin by the alkali formed. The legumin is derived from the peaflour and although said to be itself soluble, its salts are considered to be insoluble (Osborne, 1909). The above features are well seen in Fig. 1, which is a single colony, grown on a plate of trypagar, of a South African strain of type I (S.A. (I)), and also in Fig. 7, which illustrates a colony of each of the four types.

Type II is well contrasted with this. It grows as a slightly smaller round colony with a much more even edge. The surface is very smooth and glistening. No papillæ are produced. The colony soon begins to show

evidence of the development of a yellow pigment which tends to deepen in colour as the colony attains its full size. This characteristic alone is usually sufficient to distinguish it from types I and III. No halo is formed.

Type III resembles type I in many respects. The colour of its colony is whitish, or sometimes pinkish in the thick parts. The characteristic appearance of its surface may be described as "lumpy," which is well portrayed in Fig. 2. Papillæ usually do not develop on its surface and there is no halo. Some strains of type III, however, do show papillæ on the surface of the colony, notably a Danish strain, 15 (III), in my collection (Fig. 3). Such a strain seems to be an intermediate one between typical examples of types I and III.

Type IV greatly resembles type II—in fact there are practically no distinguishing marks in the colonies of these two types. Wadsworth, Gilbert and Hutton (1920) suggest that type IV, may not be a separate group, but possibly a poorly agglutinating II or III. They made some agglutinating serum from a strain of type IV obtained from Gordon which agglutinated strains of type II much more than its homologous strain. At all events there seems to be considerably less difference between types II and IV than between types I and III.

It may be useful to tabulate the above characteristics of the different types.

TABLE IV.—*Appearance of Colonies of the Different Types on Thick Trypagar Plates.*

Type.	Shape.	Size.	Colour.	Surface.	Papillæ.	Halo.
I	Irregular	Larger	Whitish or pale yellowish	Rough	+	+
II	Round	Smaller	Yellow	Smooth, glistening	0	0
III	Irregular	Larger	Whitish or pinkish	Lumpy	+ or 0	0
IV	Round	Smaller	Yellow	Smooth, glistening	0	0

As stated above, the development of these characteristics depends upon the colony having sufficient space for its complete evolution. Fig. 4 illustrates this point very distinctly. Colonies of W.30.B. (I) and Pugh (II) have been planted on the usual thick trypagar plate but they are more crowded than before, with the result that growth has been checked from lack of nourishment before the distinguishing features (rough surface, papillæ and halo in type I) could develop. The yellow pigment in the case of type II, however, could be detected, as this is an early character to appear.

On thick glucose trypsin-broth agar the pigment formation is more marked. Colonies of type I are usually pure white; type II assumes a deep yellow or even reddish-brown tint; type III had a pinkish or purplish tint; and type IV much resembles type II, although it is possibly more inclined to be yellow. Fig. 5 shows the appearance of the four types on this medium. The almost snow-white colour of S.A. (I) can be visualised from the photograph. Contrary to what occurs on trypagar, it will be observed that Pugh (II) develops papillæ

on this medium probably in response to some decomposition product of the fermented glucose.

On ordinary trypsin-broth agar papillæ do not occur on the colonies of any of the types, nor does type I produce a halo, which is in accordance with the hypothesis propounded above. There is, however, some difference between types I and III and types II and IV as a glance at Fig. 6 will show. The colonies have much the same appearance on serum agar.

It is not insisted that the characteristic appearances defined for the different types of meningococcus form hard and fast boundaries between them. It would indeed be remarkable if this were so in organisms which must be so closely related as the types of meningococcus. Thus a few papillæ may very occasionally form on type II colonies growing on trypagar. The halo described appears to be a diffusion outwards of a turbidity regularly occurring in the medium directly beneath the colonies of all the types. In plate cultures which had been stored for a week or two in the cold room prior to being photographed, it was noticed that even Pugh (II) sometimes showed a trace of a halo; the storing had afforded sufficient time for the smaller amount of alkali, or whatever substance produces the turbidity, to diffuse. The formation of the halo in the case of type I is therefore rather a difference of degree than of kind.

Two strains, both foreign ones, diverge considerably from the others. We will deal first with Sp 38. This is a Danish strain received in the early part of this year (1923) from the State Serum Institute, Copenhagen. It is said to be the most common pathogenic type in Denmark, and is a representative of Wulff's (1921) type A. It is also said not to agglutinate with any of the Gordon type sera. I found, however, that it is partly agglutinated by a type II serum when the latter is employed at × 20 titre strength. In its length of life on trypagar it conforms to type I (see Table II). The colony on thick trypagar plates is yellow like type II, but numerous papillæ are sometimes, but not always formed. A slight halo is produced. The other strain, 46 B is an American strain, and is one used by Wadsworth (1920). It agglutinates well with type II serum at titre strength but it agrees with Sp 38 in its relatively short life on trypagar. It also forms a yellow pigment, and papillæ frequently occur on the surface of the colony. There is a slight halo. One mark of distinction is that the colony is always decidedly smaller than those of other strains I have worked with. These two strains seem to possess several features in common, and 46 B appears definitely to belong to Gordon's type II even if there is some doubt about Sp 38. More than one author has expressed the view that type II represents a complex group. Thus Wadsworth, Gilbert and Hutton (*loc. cit.*) say: "It seems possible that type II is a complex group, and that its members cannot be properly differentiated by the agglutination reaction, although strains which react typically may be classified in it." Tulloch (1920) found type II to be a more complex group than the others, and advises that two different strains should always be used in doing the absorption test. It is thus clear that strains are met with which, although apparently falling into one or other group, do not entirely conform to type, and as time goes on it is only reasonable to suppose that more intermediate forms will be discovered. Since Gordon proposed his four types several authors have

reported strains which fail to agglutinate with any of the type agglutinating sera. Thus Fildes (1920) states: "Even among epidemic cocci (Naval), strains may occur commonly which do not belong distinctly to any of Gordon's types, but fall between them."

There seems no doubt that we shall have to continue to classify meningococci in the future, and perhaps such cultural tests as those described in this paper may supplement agglutination tests. Enough evidence has been accumulated to show that the latter test leaves much to be desired in the case of meningococcus.

CONCLUSIONS.

(1) By cultivating meningococci on plates composed of a thick layer of trypsin-broth-pea-agar, differences are obtained in the appearance of the colonies which correspond remarkably well with the broad division of meningococci into two main serological groups.

(2) Viability tests on the same medium yield a somewhat similar grouping, except that strains of type I are sharply marked off from all the others including type III, by their short life on this medium.

(3) These features may be usefully employed to replace or supplement serological differentiation.

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EXPLANATION OF PLATES.

Fig. 1.—Strain S.A. (I) on thick trypagar. Note the papillæ and halo.

Fig. 2.—Alternate colonies of (1) strain Pugh (II) and (2) strain C.6 (III) on thick trypagar.

Fig. 3.—Alternate colonies of (1) strain Pugh (II) and (2) strain 15 (III) on thick trypagar.

Fig. 4.—Alternate colonies of (1) strain W.30 B. (I) and (2) strain Pugh (II) on thick trypagar. Crowded.

Fig. 5.—Strains on thick glucose agar. 1 = strain S.A. (I); 2 = strain Pugh (II); 3 = strain Chase (III); 4 = strain Taplow (IV).

Fig. 6.—Strains on thick ordinary agar. Numbers as in Fig. 5.

Fig. 7.—Strains on thick trypagar containing phenol red. Numbers as in Fig. 5.

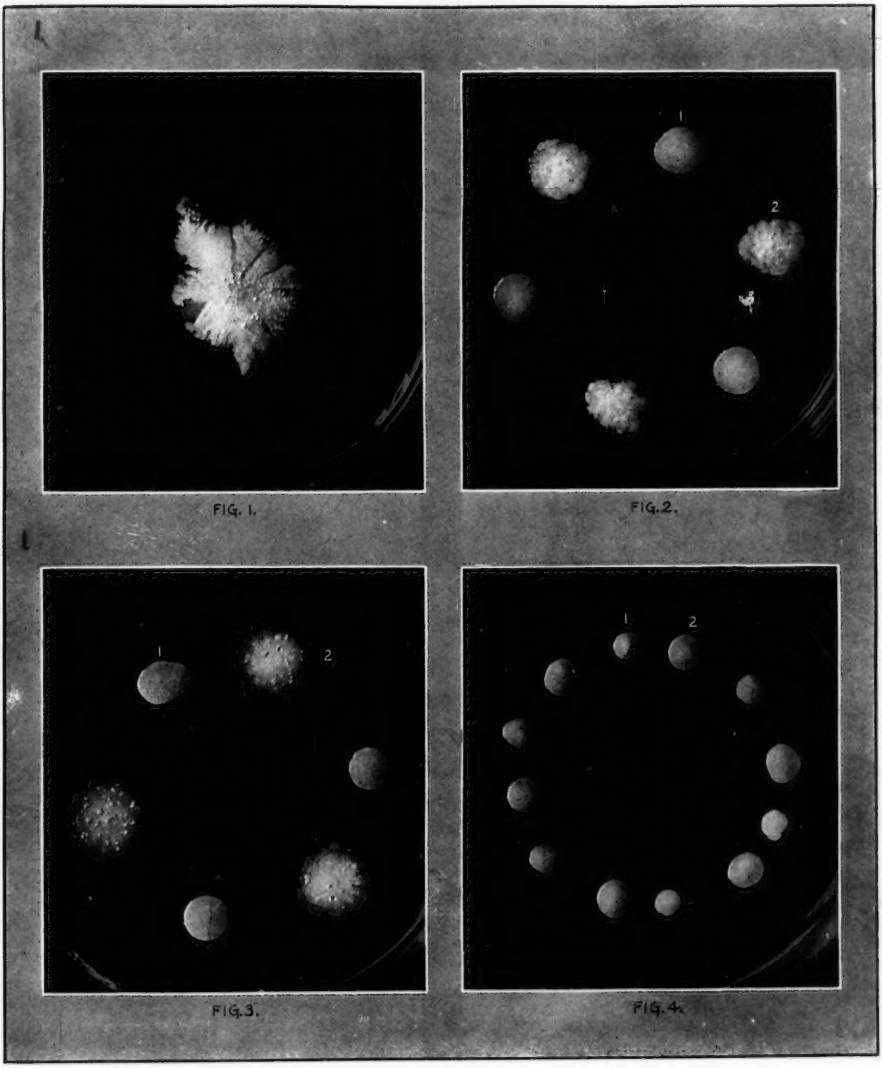

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

Atkin. Plate I.

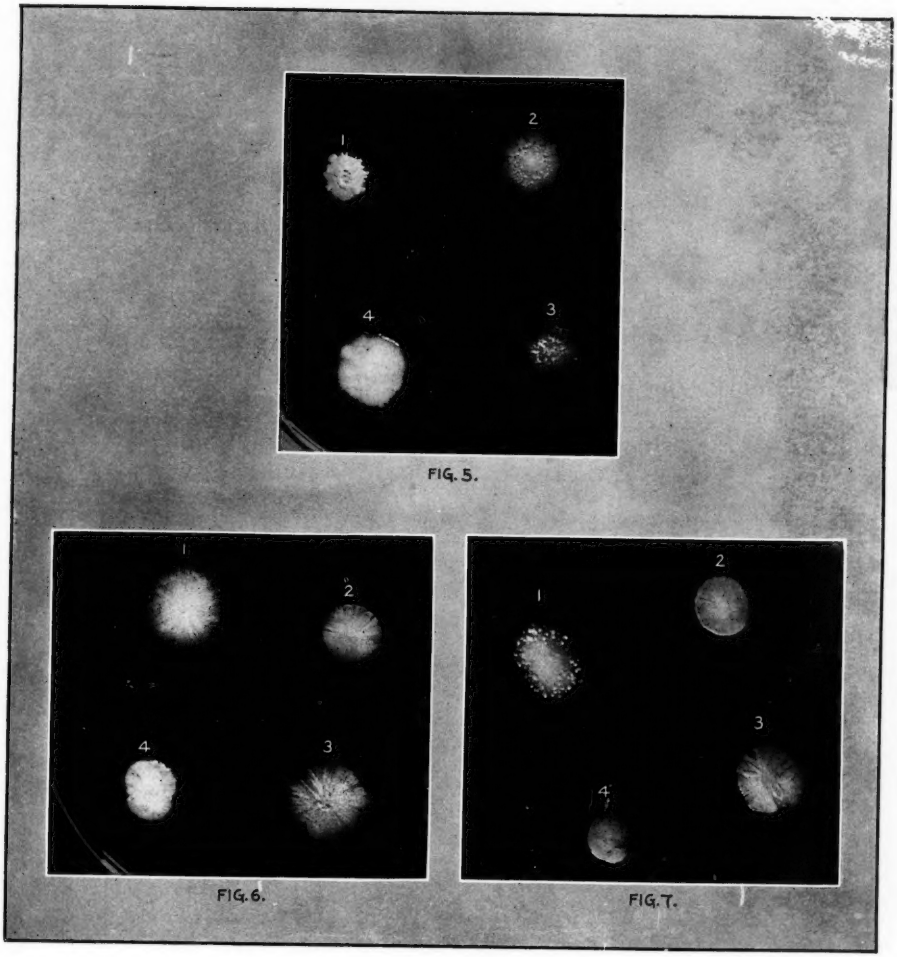

FIG. 5.

FIG. 6.

FIG. 7.

Atkin. Plate II.

THE THYROID GLAND IN EXPERIMENTAL RICKETS.

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THAT definite changes take place in the thyroid gland in certain pathological conditions has been repeatedly demonstrated. Simpson (1906) was one of the first to describe changes in some acute diseases, while Marine and Lenhart (1909) published a report on the examination of 75 thyroids from various conditions and noted marked alteration in the microscopical appearances. Farrant (1913) found that hyperplasia similar to that seen in exophthalmic goitre occurred in such diseases as infantile diarrhœa, measles, diphtheria and whooping-cough, and could be produced experimentally by the injection into animals of diphtheria toxin. Later the same author (1914) reported on the examination of 700 thyroids from as many different conditions as he could obtain them. He found that some diseases were associated with acute or complete hyperplasia of the thyroid, some with chronic or colloid hyperplasia, while others were associated with no change from the normal. Lane (1919) states that in chronic intestinal stasis the thyroid is first hypertrophied and later becomes atrophied. McCarrison (1914) in his work on experimental goitre found that keeping rats in dirty, infected cages produced an acute hyperplastic condition of the thyroid. He obtained the same appearances after subcutaneous injection of bacterial and other toxins. He has recently (1923) confirmed the former observation in pigeons, and found that the hyperplasia could be prevented by scrupulous cleanliness and to a less extent by administration of chlorine water. He states also that cod-liver oil affords complete protection against goitre produced by insanitary conditions.

A paper has just been published by Sorour (1923) giving the results of his observations on the effects of various diets which had been supposed to cause rickets in rats, and he includes a description of the thyroids. He states that the condition was not produced. He describes in some of the thyroids, particularly in those taken from rats which had been kept in darkness, a hypertrophy and microscopical appearances similar to those seen in Basedow's disease.

DESCRIPTION OF EXPERIMENTS.

The following experiments were carried out by Noël Paton and Watson in this laboratory in an investigation into the ætiology of rickets, and most of them have already been published (Paton, Findlay and Watson, 1918; Paton and Watson, 1921, 1923). Prof. Noël Paton examined the thyroids from the first experiment and noted changes in their histological appearances, which he communicated to the Physiological Society at a meeting in Edinburgh in

1920. These seemed to indicate that a routine examination of the thyroid throughout the subsequent experiments might prove of interest. Prof. Noël Paton has kindly allowed me to use the material for this, and I have examined the thyroid of as many of the animals as possible—in all, 43 pups.

Experiment.	Dog.	Thyroid.	Condition of pup.	Remarks.
I	D2	Normal	Normal	Kept in country.
	D3	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B1	Normal	Normal	Kept in country.
	B2	Commencing hyperplasia	„	Kept in country for fortnight, then in laboratory for 3 weeks.
	B3	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B4	„ „ (slight)	„	Running free in laboratory.
	B5	Acute hyperplasia	„	Confined in cage in laboratory.
II	D1	Normal	Normal	Kept in country.
	D2	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B1	„ „	„	„ „ „
	B2	Normal	Normal	Kept in country.
	B4	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B6	„ „ (slight)	„	Running free in laboratory.
	B7	ditto	„	„ „ „
VII	1	Normal	Normal	—
	2	„	„	—
	3	„	Slight rickets	—
	4	„	Normal	—
	5	„	„	—
	7	„	„	—
VIII	2	„	„	—
	3	„	„	—
	4	„	„	—
	5	„	„	—
	6	„	„	—
XVI	1	„	„	—
XVII	5	„	Rickets (healing)	Diet included olive oil.
	6	„	Rickets (healed)	Diet included olive oil, then cod-liver oil.
	8	„	Normal	Diet included olive oil.
XVIII	1	Chronic hyperplasia	„	—
	2	„ „	Rickets from Ca-starvation	Ca-deficient diet.
	3	„ „	Normal	—
	4	„ „	Rickets from Ca-starvation	Ca-deficient diet.
XIX	1	Normal	Rickets	Subdued light. Cod-liver oil after rickets developed.
	4	„	Normal	Subdued light.
XX	1	Chronic hyperplasia	Rickets	—
	2	„ „	„	Olive oil added to diet.
	3	Acute hyperplasia	„	„ „ „
XXI	1	„ „	„	Ca-free diet.
	2	„ „	„	Calcium lactate added to diet.
	3	„ „	„	Calcium sulphate added to diet.
	4	„ „	„	Ca-free diet.
	5	„ „	„	Calcium carbonate added to diet.

Full details of these experiments as regards the development or non-development of rickets may be found in the papers referred to above, so a very short *résumé* here will suffice. Experiments I and II had for their object the demonstration of the effect of confinement in the laboratory in contrast to controls of the same litter kept in the country. The latter were normal, while all the others developed rickets, although those pups which were allowed to run free in the laboratory were affected in a milder degree than those confined in cages.

Experiments VII and VIII were performed to discover what effect a diet poor in milk fat had on the occurrence of rickets. Only one of this series showed any sign of the condition, and that of a very mild degree. Dog XVI, 1 was one of a series of eight controls for Experiments XVII and XIX, being of the same litters. The former experiment was an investigation into the part played by olive oil in the production of the disease, while the latter was to find the effect of subdued light. In this series three of the pups whose thyroids I examined developed rickets. Two of these received cod-liver oil and recovered completely, while the other improved spontaneously to such an extent that the histological report on the costo-chondral junctions was given as "? early rickets." Experiment XX was a repetition of Experiment XVII, but here all the pups showed signs of rickets, although pups 1 and 2 remained healthy till almost the end of the experiment, while pup 3 developed the condition early, and was so ill that it had to be killed three weeks before the others.

Experiments XVIII and XXI were to investigate the effect of calcium starvation. The two pups in Experiment XVIII on a Ca-deficient diet developed a rachitic condition, which, however, should perhaps not be looked upon as true rickets. In accordance with Korenchevsky's suggestion (1922) I have noted this condition in the table as "rickets from Ca-starvation." In Experiment XXI all the pups became very rickety, although three of them were receiving calcium salts added to their diet. Their condition may be regarded as true experimental rickets which abundance of Ca was unable to prevent.

DESCRIPTION OF THE THYROIDS.

In the accompanying table the condition of the thyroids and the results of the experiments are given. It will be noted that the thyroids fall into three groups—normal, acute hyperplasia, and chronic hyperplasia. I have adopted these terms from Farrant's paper already quoted, as the appearances he describes under these names agree so closely with those in the pups of the above experiments.

Acute or complete hyperplasia (Fig. 2).—Such vesicles as are evident are empty of colloid, and very irregular in outline. Between these is a mass of cells, partly formed of collapsed vesicles hardly recognisable as such, and partly by proliferated cells. The vesicles are lined by cells definitely columnar in shape. The vascularity is greater than normal. Fig. 1 is from a normal pup.

Some thyroids which undoubtedly fall into this group present appearances less advanced (Fig. 3). These are marked "slight" in the table, and it is noteworthy that they are all taken from pups of Experiments I and II which were running free in the laboratory, and which, although showing signs of rickets, were affected to a less degree than those confined.

Chronic or colloid hyperplasia (Fig. 4).—The vesicles are well filled with colloid but have irregular shapes. The walls are often made up of two or three layers of cells instead of the normal single row. There are numerous invaginations of the wall into the lumen of the vesicle, the cells lining which, however, being cubical. In general appearances this type resembles the acute, with the main difference that the vesicles are filled with well-stained colloid.

Pups 1 and 2 of Experiment XX showed this condition. They had only very recently developed rickets, while No. 3, which had suffered from the disease for some time, showed acute hyperplasia. All the pups in Experiment XVIII had similar thyroids, although none had developed *true* rickets as mentioned above. It seems possible that they had been affected in a fashion similar to, but milder than, those which developed acute hyperplasia, and it is conceivable that they might have become rachitic had they been allowed to live longer.

DISCUSSION OF RESULTS.

A cursory examination of the table shows that of the 43 pups under review every case of rickets was associated with acute hyperplasia of the thyroid. In Experiments I, II and XXI the changes in that gland were very marked, but, as pointed out above, varied with the severity of the rickets. In Experiments VII and VIII, where only one very mild case of rickets occurred, the thyroids were normal.

Quite definite rickets developed in the pups of Experiments XVII and XIX, but they all improved before they were killed, especially those which got cod-liver oil, which recovered completely. It is interesting to note that the thyroids in these cases were normal. That cod-liver oil has a beneficial action on rickets has long been an accepted fact, but its curative effect on experimental goitre has only recently been demonstrated by McCarrison. In these experiments the cod-liver oil definitely improved the rickets, and presumably, as all pups with acute rickets had hyperplastic thyroids, it restored that gland to normal.

I have found that cod-liver oil has a marked influence on the colloid content of the thyroid vesicles. Rats of the same litter were kept under exactly similar conditions and fed on a carbohydrate diet. Half of the rats received cod-liver oil in addition. The thyroids of the latter were made up of vesicles well filled with colloid (Fig. 5), while those getting no oil had glands with small empty vesicles (Fig. 6). Butter-fat was tried in place of cod-liver oil but was found to have no effect, the thyroids of the animals receiving it and those of the controls having small empty vesicles as in the controls of the other experiment. This condition is similar to that described as hyperplasia and is apparently prevented by the administration of cod-liver oil.

Mellanby (1921) has found similar appearances in the thyroids of animals fed by him on various fats, but he interprets them in a different way. He found that animals fed on a diet containing cod-liver oil had normal thyroids, while those getting butter-fat showed hyperplasia. He considers the butter-fat as the causal agent of the hyperplasia, but if my suggestion is right it would appear that the true cause was probably infective or toxic in nature, from which the others were protected by the cod-liver oil.

Considering how closely these changes in the thyroid in acute experimental

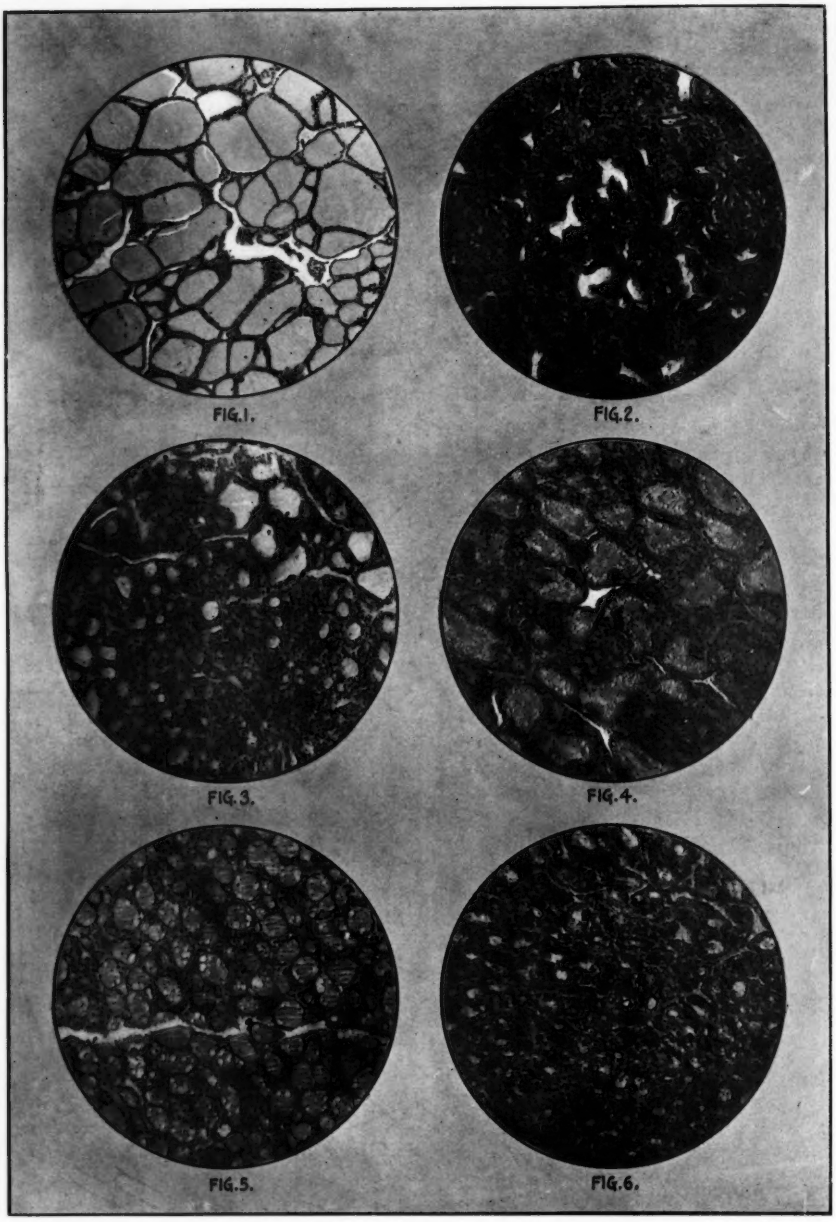

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

rickets agree with those described by Farrant in various diseases, it seems possible that here, too, they are the result of a toxæmia. That such a state should occur in these pups which developed rickets is not inconceivable when one considers the experiments carefully. Whatever the apparent cause of the disease, be it confinement, poor nutrition, or subdued light, it is obvious that the adverse conditions to which the animal was subjected would be most likely to favour the development of some infection. In Experiment XX one of the pups was suffering from some such condition—so much so in fact that it had to be killed before the others of the litter—and it was in this animal that most marked rickets and thyroid changes were observed.

There has been a considerable amount of work done by various observers in an attempt to prove the "infective theory" of rickets, but so far it has not been conclusive. It is interesting to find in the examination of these thyroids what might be called presumptive evidence in favour of this theory.

CONCLUSIONS.

1. With the occurrence of rickets in pups there are changes in the thyroid resembling those seen in some infections.

2. These observations show that the rachitic-like changes of Ca-deficiency are not associated with the changes in the thyroid which occur in what may be called true rickets.

3. True rickets may attack pups on a Ca-rich diet as shown by Experiment XXI.

4. Cod-liver oil acts on the thyroid in some way either by preventing destruction or increasing production of colloid.

My sincere thanks are due to Prof. Noël Paton for kindly help during the prosecution of the work, and to Mr. Bell, who prepared the photographs.

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DESCRIPTION OF FIGURES IN PLATE.

Fig. 1.—Dog's thyroid, normal.
Fig. 2.—Dog's thyroid, showing acute hyperplasia.
Fig. 3.—Dog's thyroid, showing acute hyperplasia of a milder degree.
Fig. 4.—Dog's thyroid, showing chronic hyperplasia.
Fig. 5.—Thyroid of rat which had cod-liver oil added to its diet.
Fig. 6.—Thyroid of control rat receiving no fat of any kind.

VARIATIONS IN THE DISTRIBUTION OF CO_2, CHLORINE AND CALCIUM IN THE CELLS AND PLASMA OF THE BLOOD IN TETANY.

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THAT there exists a relationship between tissue irritability and anion concentration in the blood has been demonstrated by several investigators (MacCallum, Loeb, Mathews and Tisdall). That the tissue irritability which is indicative of a condition of tetany is related in some way to the degree of ionisation of calcium has also been determined experimentally (Cruickshank, 1923[1]).

In a recent paper (1923[2]) the author has shown that there are two distinctive phases in the development of tetany, one a non-toxic phase, in which the associated blood condition is one of alkalosis, the other a distinctly toxic condition, in which acidosis is marked. That these two phases may play a part in determining the degree of ionisation of the divalent salts is a possibility worth bearing in mind. Freudenberg and György maintained in 1913 that the free calcium in the blood was in equilibration with the bound calcium in the tissues, and suggested that tetany was due to the fact that a loss from the blood of free calcium resulted in a withdrawal of calcium from muscle and nerve tissue. In 1920 they demonstrated that an increase in the pH of the blood would result in an increase in combined calcium, and in the same year Collip (1920) showed that in alkaline solution calcium salts are less ionised than they are in acid solutions. It would seem; therefore, a possibility that the condition of acute tetany is one in which the pH is lowered, accompanied by a decided loss of calcium from the tissues; and further, it would appear that the condition is due not so much to a loss of calcium in the blood bathing the tissues, though that does obtain, but rather to a loss of combined calcium, which renders the tissues more prone to the toxic effects of the irritant monovalent salts.

But while experimental evidence is brought forward to show variations in the amounts of ionic and colloidal salts in the blood, these variations should at present be regarded as concomitant changes due to a causative factor rather than being *per se* the cause of the acute condition following thyroparathyroidectomy in animals.

MacCallum (1920) and his co-workers have brought forward evidence that in tetany produced by pyloric obstruction there is an increase in the CO_2-combining power of the plasma and a decrease in its chloride content.

Hastings, Murray and Murray (1921) corroborate this, with the additional note that the calcium content of the plasma is slightly increased. Tisdall (1922[2]), who has made a more detailed investigation of gastric tetany in adults, found that the calcium content was normal, namely 10·6 mg. per cent., the chlorides had decreased from 360 mg. to 215 mg., the sodium had fallen from 353 mg. to 287 mg., while the CO_2-combining power had risen from 65 to 103 vols. per cent. It is apparent, then, that the CO_2-combining power of the blood and the sodium : calcium ratio are disturbed in the opposite direction in gastric tetany to that which obtains in parathyroid and other types of tetany. In parathyroid tetany the usual results have been a fall in calcium from a general average of 10·0 mg. to that of 6·0 mg. per cent. in whole blood, and a quite definite reduction in the sodium and in the CO_2-combining power of the blood, these changes being accompanied by a lowering of the pH of the blood.

It is known that in the maintenance of normal function the ratio of the inorganic ions of the blood is of far more importance than are changes in the absolute amounts of each. The part played by inorganic salts in causing infantile tetany has been investigated by Kramer, Tisdall and Howland (1921); they found that calcium was markedly diminished, while the phosphates and magnesium remained practically constant; they make no reference, however, to chlorides.

The nature of the alterations in the inorganic salts of the blood in parathyroid tetany has been more fully studied by Gross and Underhill (1922). It should be noted, however, that the estimations made are of the variations in the elements as such, and not of the variations in the amounts of these elements present in the ionic as opposed to the colloidal state. Such variations can be accurately determined by the method of compensation dialysis as has been done for calcium (Cruickshank, 1923[1]). The average values for the inorganic elements in normal animals estimated by Gross and Underhill in milligrammes per 100 grammes by weight of whole blood are Cl 310·0, total P 36·0, Ca 5·6, K 30·0, Na 310, Mg 5·5. For tetany animals they obtained results which show a marked change in certain of the inorganic elements of the blood, namely, a fall in chlorides of 30 mg. and in calcium of 4·0 mg. per 100 gm. of whole blood, while the sodium and magnesium content remained constant or only fell slightly and the phosphorus and potassium increased by an average figure of 10·0 and 5·0 mg. respectively. The important point here is not those absolute amounts but the variation in certain ratios.

That calcium varies directly with the H-ion concentration in physico-chemical mixtures of calcium and carbonate was first suggested by Rona and Takahashi (1913). If the H-ion concentration of the mixture remains constant then the Ca-ions and HCO_3-ions will vary in inverse proportion, or if the H-ion concentration increases, then the Ca-ion will increase in proportion to the diminution of the carbonate in accordance with the equation,

$$\frac{[Ca] \times [HCO_3]}{[H]} = K.$$

In 1915 Loeb found that the ratio of the sum of the monovalent elements to the sum of the divalent elements must be maintained constant in order to preserve normal irritability of nerves. Calculation from the figures of Gross

and Underhill of the ratio of monovalent to divalent elements indicates that such a ratio approaches a constant figure. The average figure for the ratio of $\frac{(Na) + (K)}{(Ca) + (Mg)}$ is 31·25; other ratios that are of importance are that of Na : Ca and K : Ca. Tisdall regards the former of importance, while Gross and Underhill seem to regard the latter with that of P : Ca of greater significance. They have shown that in parathyroid tetany the $\frac{(Na) + (K)}{(Ca) + (Mg)}$ ratio changes from the average figure of 31·5 to one of 46·0, while the ratio of K : Ca changes from an average of 5·1 to one of 18·5. Knowing that sodium and potassium salts act as irritants to nervous tissue, and that any increase of these in the blood-stream will precipitate or aggravate an attack of tetany, and that the action of the divalent salts is diametrically the opposite, one can understand the possible importance of a knowledge of these ratios in the treatment of the disease. While such ratios are of interest and value, what is of greater value is a knowledge of the degree to which the colloidal condition of the various elements has been disturbed, and some indication as to alterations in the distribution of these elements.

As to the former, namely, alterations in the ionic and colloidal state of the elements, evidence has been brought forward with regard to calcium which shows that there is an almost complete breakdown of colloidal calcium in acute tetany—an event which is in agreement with those *in vitro* experiments which show that any increase in H-ion concentration of solutions of calcium salts is associated with a liberation of Ca-ions. As to whether or not the cell membrane has suffered damage and thereby become more permeable to ions, or whether the cellular function has been disturbed so that ions are lost, must be investigated both by dialysis and a determination of the distribution of these ions in the cells and in the plasma.

With regard to the chlorides, it has been indicated that there may or may not be a definite fall in the chlorides of the whole blood. It will be seen from Table I that during the first stage of tetany there is usually no marked alteration in the chloride content of the plasma and corpuscles, but that, with the development of acute tetany, the animals showing all the signs of a severe toxic condition, there is a loss of chloride from the cells, and further that the loss increases up to some 40 per cent. of the total cell chloride. It has also been shown that this loss may run almost parallel with the diminution in the alkali reserve of the blood, which may amount to some 60 per cent. of the normal. The CO_2-combining power of the blood is lowered, but the most marked loss of CO_2 is that which occurs from the cells. These results would seem to indicate that the tetany toxin is of the nature of a non-volatile acid, which depletes the alkali reserve and attacks the hæmoglobin molecule, reducing its base and thus liberating Cl-ions, which, however, do not increase the chlorine cellular content. The loss of chlorine from the cell is so marked, amounting from 10 to 40 per cent. of its total chloride content, that it seems justifiable to assume that the tetany poison has damaged to a considerable extent the corpuscular economy, thereby liberating Cl-ions from their colloidal combination.

TABLE I.

	CO_2.				Chlorides.						
	Blood.	Plasma.	Cells.	Cell percent. of total	Blood.	Plasma.	Cells.	Cell percent. of total.	Hæmatocrit.	pH.	Remarks.
1*a*	43·912	33·085	10·872	24·6	·625	·450	·175	28·0	3·5	7·40	Normal.
b	48·110	35·59	12·52	26·5	·615	·438	·177	28·8	3·2	7·45	T. mild.
c	26·298	21·515	4·653	17·9	·670	·514	·156	23·3	3·5	7·25	T. acute.
	40·8	34·5	57·4	—	8·8	(14·0+)	18·5	—	—	—	Percent. loss.
2*a*	46·65	33·82	12·53	26·8	·505	·375	·130	25·70	4·0	7·35	Normal.
b	42·355	32·780	9·575	20·1	·540	·424	·116	21·50	3·6	7·40	T. mild.
c	35·964	28·718	7·186	20·0	·510	·391	·119	32·30	3·7	7·30	T. acute.
	22·9	15·5	42·7	—	0·99	(4·27+)	8·46	—	—	—	Percent. loss.
3*a*	40·836	29·77	11·066	27·10	·650	·482	·168	25·86	3·2	7·35	Normal.
b	43·884	38·197	5·687	12·96	·595	·504	·091	18·85	2·35	7·30	T. definite.
c	39·606	34·00	5·606	14·15	·620	·528	·092	17·90	2·0	7·25	T. acute.
	3·0	—	49·3	—	4·6	(9·5+)	39·3	—	—	—	Percent. loss.
4*a*	42·50	32·45	10·04	23·5	·615	·462	·153	24·9	3·0	7·35	Normal.
b	40·098	32·890	7·208	18·0	·570	·448	·122	21·4	3·0	7·45	T. definite.
c	38·560	30·366	8·194	21·2	·535	·444	·091	17·0	2·95	7·35	T. acute.
	90·7	3·36	18·45	—	13·00	3·9	40·60	—	—	—	Percent. loss.

TABLE II.

	Per cent. in whole blood.	Per cent. in plasma.	Plasma per cent. of whole.	Per cent. in cell.	Cell per cent. of whole.	Remarks.
Calcium	9·12 mg	8·11 mg.	88·9	1·01 mg.	11·1	Normal.
	5·72 „	5·26 „	91·9	0·46 „	8·1	Acute tetany.
	37·40	35·20	—	54·4	—	Per cent. change.
Chlorine	·599 gm.	·442 gm.	73·9	·156 gm.	26·1	Normal.
	·563 „	·449 „	79·8	·114 „	20·2	Acute tetany.
	6·1	±	—	26·91	—	Per cent. change.
CO_2	43·45 vols.	32·25 vols.	74·20	11·20 vols.	25·8	Normal.
	35·1 „	28·60 „	81·5	6·50 „	18·5	Acute tetany.
	19·20	11·31	—	41·9	—	Per cent. change.

Changes in: $[CO_2]$.	$[BHCO_3]$.	Alk. reserve.	pH.	Remarks.
18·39	17·25	17·25	7·37	Normal.
13·05	12·26	5·26	7·31	Acute tetany.
29·00	29·40	30·18	8·15	Per cent. change.

TABLE III.—*Equilibration Data from Blood of Normal Dog.*

Atmosphere.		Blood.					
CO_2 per cent.	pCO_2.	CO_2 per cent.	$[CO_2]$.	$[H_2CO_3]$.	$[BHCO_3]$.	R.	pH.	
1·92	13·44	24·45	10·82	·4032	10·42	25·90	7·593	—
3·97	27·79	34·56	15·43	·8337	14·60	17·54	7·424	—
5·81	40·67	39·90	18·00	1·2201	16·78	13·75	7·318	—
10·23	71·61	49·31	22·00	2·1485	19·85	9·26	7·147	—

Equilibration Data from Blood of Tetany Dog.

CO_2 per cent.	pCO_2	CO_2 per cent.	$[CO_2]$.	$[H_2CO_3]$.	$[BHCO_3]$.	R.	pH.	
2·68	18·90	18·78	8·39	·5670	7·83	14·06	7·327	—
3·43	24·20	21·47	9·58	·7260	8·86	12·20	7·266	—
6·50	45·80	29·50	13·17	1·3740	11·80	8·60	7·114	—
11·30	79·75	39·30	17·56	2·3925	15·17	6·35	6·980	—
3·904	(27·33)	35·95	16·05	(0·820)	(15·13)	15·22	(7·39)	Normal.
3·595	(95·33)	22·30	9·96	(0·760)	(9·20)	12·02	(7·26)	Tetany.
—	—	—	6·80	—	6·42	—	7·39	At normal pH.

Change in alkali reserve—8·71 mm. $BHCO_3$.

Note.—Figures in brackets are obtained by interpolation on the curve obtained from equilibration data from the blood of normal and tetany dogs. $R = \frac{BHCO_3}{H_2CO_3}$. $pH = \log R + pK_1$. $pK_1 = 6·18$.

In Table II are given the average results of experiments made to determine the distribution of calcium, chlorine and CO_2 in the plasma and cells and alterations in the bicarbonate content and alkali reserve of the whole blood of normal and tetany dogs. The $[BHCO_3]$ content of the blood was determined from equilibration data as shown in Table III. To determine accurately the changes in alkali reserve it is necessary to extrapolate the $[BHCO_3]$ value of the tetany blood to that which would obtain for tetany blood under a CO_2 tension which would restore the normal pH. The average figures given in Table II show that in acute tetany, so far as the blood is concerned, the alterations in calcium, chlorine and CO_2 are associated with some disturbance of intracellular function. That such a loss in alkali reserve as is shown here should bear a relationship to the loss of chlorine from the blood-cells while the chlorine content of the plasma remains almost stationary would indicate a definite alteration in the manner of the combination of alkali and oxygen by the hæmoglobin molecule. To determine the nature of the damage within the cell one would require to know what changes are occasioned in the ionic and colloidal contents of the cellular elements, and for this a method for the estimation of these forms in the corpuscle is still wanting.

CONCLUSIONS.

In the early stages of tetany there is usually no pronounced change in the chloride content of either corpuscles or plasma. In later stages, and particularly when toxic symptoms appear, there is a reduction of chloride, especially in the corpuscles, which may lose from 10 to 40 per cent. of their chloride. Accompanying and probably directly related to this is a fall in the alkali reserve, particularly of the corpuscles, and a reduction of the calcium content of both corpuscles and plasma. It is suggested that the toxic agent in tetany is a fixed acid.

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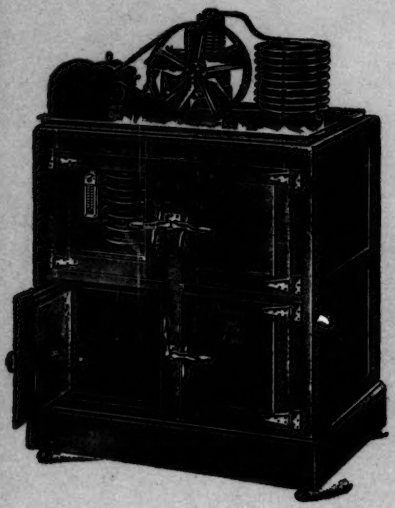

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